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Development of in Vivo Biomarkers for Progressive Tau Pathology after Traumatic Brain Injury

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14. ABSTRACT

Athletes in contact sports who have sustained multiple concussive traumatic brain injuries are at high risk for delayed, progressive neurological and psychiatric deterioration [1-9]. This syndrome is termed chronic traumatic encephalopathy (CTE) [1, 7, 10], and is also known as dementia pugilistica [3, 11] or 'punch drunk' syndrome [9, 12]. US military personnel [13, 14] and others who have sustained multiple concussive traumatic brain injuries [15-17] may also be at risk for this condition. Currently, there are no methods to identify progressive tau pathology in living humans. **Hypothesis:** Aggregated forms of hyperphosphorylated tau protein formed acutely in the setting of traumatic brain injury can seed further aggregation of intracellular tau in nearby cells, leading to delayed propagation of tau pathology and neurodegeneration. **Objective:** To develop standardized, high-throughput blood and cerebrospinal fluid assays for aggregated forms of tau responsible for propagation of tau pathology after traumatic brain injury.

Progress to date: To date, none of the attempts to model progressive tau pathology after repetitive concussive TBI in mice has been optimal. Ongoing efforts include development rotational acceleration concussive injury which can be repeated 20 times in the same mice, and subconcussive injuries. Continued progress towards increasing the sensitivity of cell-based assays for tau aggregation activity has been made, as well as antibody-based tau detection methods for blood samples.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION: Athletes in contact sports who have sustained multiple concussive traumatic brain injuries are at high risk for delayed, progressive neurological and psychiatric deterioration [1-9]. This syndrome is termed chronic traumatic encephalopathy (CTE) [1, 7, 10], and is also known as dementia pugilistica [3, 11] or 'punch drunk' syndrome [9, 12]. US military personnel [13, 14] and others who have sustained multiple concussive traumatic brain injuries [15-17] may also be at risk for this condition. Hyperphosphorylation and aggregation of tau protein are key pathological features of chronic traumatic encephalopathy, but at present they can only be observed post-mortem [1, 3, 6, 18-20]. Tau pathology has also been observed after single more severe traumatic brain injuries [21-23]. Currently, there are no methods to identify progressive tau pathology in living humans. The progressive aspect of chronic traumatic encephalopathy suggests that repetitive injuries may trigger an ongoing degenerative process similar to other diseases characterized by progressive tau pathology such as Alzheimer disease and frontotemporal dementia. A leading hypothesis regarding the progression of tau pathology in Alzheimer disease and frontotemporal dementia is that tau aggregates formed in one cell can propagate by exiting that cell and entering anatomically connected cells to induce tau aggregation in these cells [24-30]. While the tau pathology in chronic traumatic encephalopathy is distinct from other diseases, the propagation model offers a new conceptual framework to test these ideas in chronic traumatic encephalopathy.

Hypothesis: Aggregated forms of hyperphosphorylated tau protein formed acutely in the setting of traumatic brain injury can seed further aggregation of intracellular tau in nearby cells, leading to delayed propagation of tau pathology and neurodegeneration.

Objective: To develop standardized, high-throughput blood and cerebrospinal fluid assays for aggregated forms of tau responsible for propagation of tau pathology after traumatic brain injury.

The Specific Aims for the Brody and Diamond labs, taken together were:

TASK 1: To assess extracts from the brains of tau transgenic mice subjected to experimental traumatic brain injury for tau aggregating activity using a cultured-cell based assay.

TASK 2: To determine whether mouse blood and cerebrospinal fluid tau aggregating activity quantitatively predict brain tau pathology and neurodegeneration in mice subjected to experimental traumatic brain injury

TASK 3: To test whether antibodies that block tau aggregating activity in cultured cell-based assays also block tau pathology, neurodegeneration and behavioral deficits in mice subjected to experimental traumatic brain injury

TASK 4: To develop an antibody-based assay for tau aggregating activity

This report includes only the work performed by the Brody lab.
Because of no-cost extensions relating to the Diamond lab move to UT Southwestern, the Diamond lab work is still ongoing,

BODY
PROGRESS DURING THE REPORTING PERIOD:

Brody Laboratory
The major challenge encountered was to reliably recapitulate progressive tau pathology triggered by repetitive concussive TBI in an animal model.

We took multiple novel approaches to the problem:

A) We tested the effects of 2 impact concussive injuries in wild-type (non-mutated) human tau expressing mice (hTau), but found no effect on tau pathology 7 days after injury. The concussive injuries were performed appropriately, as validated by increased silver staining in gray and white matter, indicative of injury. [2014 Annual Report]
B) We tested the effects of 4 daily impact concussive injuries in hTau mice, but found no effect on tau pathology 7 days after the last injury. [2014 Annual Report]
C) We tested the effects of more severe contusional TBI on 3xTg-AD mice expressing a rare mutant form of tau associated with familial fronto-temporal dementia. These more severe injuries caused an increase in tau immunoreactivity at 1 day and 7 days after injury, but 6 months after injury this tau pathology had largely resolved. [2014 Annual Report]
D) We tested the effects of 4 daily impact concussive injuries in P301S mice expressing a different mutant form of tau associated with familial fronto-temporal dementia. There was no effect on tau pathology. There was no effect of injury on tau prion-like seeding activity in brain lysates, blood or cerebrospinal fluid at 7 days, 6 weeks and 3 months after injury. [2014 Annual Report]
E) We tested the effects of 4 daily impact concussive injuries in hTau mice, but found no effect on tau pathology 6 months after the last injury. We tested an ultrasensitive immunofluorescent method involving array tomography and still did not find any evidence of increased tau pathology. [2015 Annual Report]
F) We tested the effects of combined daily high dose ethanol administration and 4 daily impact concussive injuries in 12 month old hTau mice. We found no effect of injury, ethanol administration or combined injury and ethanol on tau pathology 6 months after the last injury. [2016 Annual Report]
G) We tested the effects of 4 daily rotational acceleration (CHIMERA) concussive injuries on P301S mice and found inconsistent effects on tau pathology.
H) We tested the effects of 20 daily rotational acceleration concussive injuries on hTau mice and found no effects on tau pathology at 12 months after injury. There was clear evidence of behavioral dysfunction and axonal disruption in white matter, indicating that the injuries were performed effectively. Testing for brain tissue lysate, blood and cerebrospinal fluid tau prion-like seeding activity is ongoing in the Diamond lab. [2017 Quarterly Reports and Manuscript in Preparation, attached as an appendix.]

BIBLIOGRAPHY OF MEETING ABSTRACTS AND PUBLICATIONS

1) Gangolli et al, "Chronic neurobehavioral and neuropathological outcomes following repetitive head impacts in a transgenic mouse model" National Neurotrauma Society Meeting, 2017
2) Mihika Gangolli, Joseph Benetatos, Thomas J. Esparza, Emeka M. Fountain, Shamilka Seneviratne, and David L. Brody "**Repetitive concussive and subconcussive injury in a human tau mouse model results in chronic cognitive dysfunction and disruption of white matter tracts, but not tau pathology**" *manuscript in preparation*

PERSONNEL RECEIVING PAY (Brody Lab only)

David L. Brody – PI
Mihika Gangolli- PhD student
Joseph Benetatos- Technician
Thomas Esparza- Senior Scientist
Rachel Bennett- PhD student
Theodore Floros- technician

In addition, the following people contributed but did not receive pay
Emeka Fountain- Technician
Shamilka Seneviratne- Undergraduate student

CONCLUSION

It has proven surprisingly difficult to recapitulate tau pathology induced by repetitive concussive TBI in mice. Efforts in larger animal models such as ferrets and pigs are underway, and may represent a promising way forward in this important field. It is also possible that additional factors beyond repetitive concussive injuries in isolation are required to induce CTE-like tau pathology. We tested one candidate factor- ethanol consumption- but did this did not affect tau pathology. Furthermore, it is possible that CTE-like tau pathology required decades to manifest due to slow changes in conformation, aggregation, and spreading of prion-like tau; if so, this will be challenging to model in animals. Finally, recent analysis of high school and collegiate football players indicates that the number of lifetime sub-concussive impacts appears to have a stronger relationship with later life cognitive and behavioral concerns than the number of concussions[31]. The number of sub-concussive impacts required to increase risk substantially was reported to be in the thousands. Thus, creative approaches to modeling thousands of lifetime sub-concussive impacts may be required to test the hypotheses that these events are the cause of CTE-like tau pathology. At present, there is no animal model involving thousands of sub-concussive impacts to our knowledge. The model of Petraglia et al,[32, 33] involved 6 impacts per day for 7 days. If 6 impacts per day were continued for 500 days over a 2 year period, the experimental paradigm would reach 3000 lifetime impacts. Clearly this would be very labor intensive and automated methods would likely be required.

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Repetitive concussive and subconcussive injury in a human tau mouse model results in chronic cognitive dysfunction and disruption of white matter tracts, but not tau pathology

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Abstract

Due to the increasing need for a means to study chronic traumatic encephalopathy (CTE) *in vivo*, there have been numerous efforts to develop an animal model of this progressive tauopathy. However, there is currently no consensus in the field on an injury model which consistently reproduces the neuropathological and behavioral features of CTE. We have implemented a repetitive CHIMERA injury paradigm in human transgenic (hTau) mice. Animals were subjected to daily subconcussive or concussive injuries for twenty days and tested acutely, three months, and 12 months post injury for deficits in social behavior, anxiety, spatial learning and memory, and depressive behavior. Animals were also assessed for chronic tau pathology, astrogliosis, and white matter degeneration. Repetitive concussive injury caused acute deficits in Morris water maze performance, including reduced swimming speed and increased swimming distance during visible and hidden platform phases, that persisted during the chronic time point following injury. We found evidence of white matter disruption in animals injured with subconcussive or concussive injuries, with the most severe disruption

occurring in the repetitive concussive injury group. Severity of white matter disruption in the corpus callosum was moderately correlated with swimming speed, while white matter disruption in the fimbria showed weak but significant correlation with worse performance during probe trial. There was no evidence of tau pathology or astrogliosis in sham or injured animals. In summary, we show that repetitive brain injury produces persistent behavioral abnormalities as late as one year post injury that may be related to chronic white matter disruption.

Key Words

Concussive, subconcussive, chronic behavior

Introduction

Chronic traumatic encephalopathy (CTE) is a progressive, neurodegenerative disease that cannot currently be diagnosed in living patients. Individuals with suspected CTE who are diagnosed post-mortem typically have a history of repeated head impacts and often display mood and depressive type symptoms, typically manifesting approximately a decade following the initial impacts, followed by late stage cognitive failure.[1-3] The distinguishing neuropathological features of CTE, including depositions of phosphorylated tau (ptau) tangles and tau positive astrocytes distributed in depths of cortical sulci and in perivascular regions, are initially observed in the superior and dorsofrontolateral prefrontal cortex. In later stages of CTE, neurofibrillary tangles (NFTs) and astroglial tau are found in the temporal lobe, hippocampus, amygdala, and inferior cortices.[4, 5] Detection of these features is the only means to provide a diagnosis of CTE, and is performed using immunohistochemical staining of brain tissue. Therefore, an animal model of CTE would provide much needed insight, not only when developing diagnostic biomarkers, but also when testing potential therapeutics *in vivo*. There have recently been numerous efforts to develop the necessary animal model of CTE using a variety of injury paradigms with mixed results (Supplemental Table 1). In wild type mice, cognitive and social deficits, accompanied by depressive-like behavior manifest acutely following injury and are present as late as six months post injury.[6-11] For example, Luo et al implemented a repetitive closed head controlled cortical impact (CCI) mode in adult mice that resulted in cognitive deficits during radial arm maze, increased ptau indicated by AT8 immunoreactivity, and astrogliosis indicated by GFAP immunoreactivity.[9] Concurrent work by Petraglia et al used an increased number and frequency of impacts in adult mice that caused increased risk taking behavior and persistent Morris Water Maze deficits along with increased AT8 and GFAP immunoreactivity at both acute and chronic time points.[7, 8] In contrast, an early study by Yoshiyama and colleagues using transgenic mice overexpressing the shortest human isoform (T44) found no significant neurobehavioral differences or histopathological evidence of ptau tangles in injured animals six months following repeated injuries.[12] More recent work found that while repetitive closed headed impacts caused progressive behavioral impairments and neuroinflammation, there was no ptau pathology at chronic time points up to six and twelve months post injury using immunohistochemistry methods.[13, 14] Furthermore, studies of the temporal dynamics of ptau showed an acute increase followed by a return to baseline at 60 days post injury in wild type mice, a finding paralleled in work by Tran et al using a single, more severe injury in triple transgenic 3xTG mice. [15, 16 17]

The inconsistency of these initial findings raised the question of whether failures of previous attempts to reproduce the chronic and progressive ptau pathology seen in humans was due to the fundamental differences between tau in the human and mouse brain. While wild type mice typically express only the 4R isoforms of tau, the human brain expresses 3R and 4R isoforms in equal amounts.[18] This provided the motivation to study the chronic effects of repetitive TBI in mice that express the tau isoforms seen in humans (hTau). These mice have been reported to develop age-associated thioflavin S neurofibrillary tangles between nine and 15 months of age, along with cognitive deficits that manifest at twelve months of age.[18, 19] Preliminary work exploring the relationship between brain injury and ptau pathology in the hTau mouse line was performed by Ojo et al, and showed that repetitive impacts in aged (18 month old) mice resulted in increased cortical ptau along with increased neuroinflammation. [20] The same group found that adult hTau mice exposed to repeated head injuries also had increased cognitive deficits along with elevated total tau levels at six months following injury.[21] These studies provide an initial insight into the role of repeated brain trauma and chronic ptau pathology but do not consistently show progressively worsening neurobehavioral deficits nor the neurofibrillary tangles and tau positive astrocytes that are pathognomonic for CTE.[14, 20, 21]

To develop an animal model of CTE, several factors must be considered: age at the onset of injuries, injury frequency and severity, number of injuries, and time from injury to assessment., Importantly, while models such as CCI have been shown to cause increased blood brain barrier (BBB) permeability the biomechanics of the injury model may need to be reconsidered and modified to incorporate increased strain and strain rates. These have been shown to lead to increased BBB disruption, resulting in tissue damage in perivascular regions.[22-24] Furthermore, implementation of a rotational acceleration injury model in gyrencephalic animals such as pigs has resulted in accumulation of tau colocalized with ßAPP and neurofilament staining indicative of axonal injury, illustrating the need to incorporate such a component in order to better mimic the brain trauma experienced during a concussive or subconcussive event.[25, 26] The CHIMERA (Closed Head Impact Model of Engineered Rotational Acceleration) is potentially advantageous for this very reason. This model combines high injury reproducibility with the ability to implement a nonsurgical impact plus rotational acceleration injury in small, lower cost animals such as mice.[27] A recent study using three 0.5J injuries has resulted in chronic cognitive deficits accompanied by increased Iba1 and GFAP levels as late as six months post injury. [28]

Furthermore, the versatility of impact thresholds at subconcussive and concussive levels has previously been tested and shows neurobehavioral and histopathological changes in the rodent brain. [29]

We have implemented a repetitive CHIMERA injury paradigm in hTau mice at two energy levels; concussive and subconcussive. The aim of the study is to determine the cumulative effects of repetitive brain trauma in adult mice (4 months of age) at acute (one month), intermediate (three months), and chronic (twelve months) time points post injury. We hypothesized that the repeated impacts plus rotational acceleration would induce tissue shearing, white matter disruption, and in the chronic phase, accumulation of cortical and perivascular neurofibrillary tangles, astrogliosis, and white matter disruption accompanied by progressive neurobehavioral deficits comparable to those exhibited by patients with a diagnosis of CTE. As part of our pre-specified plan, cerebrospinal fluid (CSF) and blood was collected prior to sacrifice for exploration of potential fluid biomarkers of chronic phase repetitive concussive TBI-related tau pathology.

Methods

Animals: Fifty hTau mice (C57BL/6 Cg-Mapt<tm1(EGFP)Klt>(MAPT), Cat #00549, Jackson Laboratory, Bar Harbor, ME, USA) aged 4-8 weeks were purchased as two cohorts of twenty five animals each. All mice were allowed to acclimate to 12 hour light-dark cycles. Food and water were supplied *ad libitum*.

CHIMERA injury and anesthesia

Injuries were performed using the CHIMERA injury method, which involves a midline closed head impact followed by rotational acceleration. Each animal was anesthetized with 5% isoflurane for 2.5 minutes followed by maintenance at 2.5% isofluorane during positioning on the platform. Total isoflurane exposure did not exceed five minutes and delivery was stopped immediately before impact. Sham animals underwent the same anaesthesia protocol and head positioning on the CHIMERA device but no impact by the piston.

Determination of injury thresholds

Because animals were exposed to twenty daily impacts, the energy levels for subconcussive and concussive impacts were determined empirically (Supplemental Figure 1A). Latency to right reflex (LRR), defined as the time from impact to regaining consciousness and becoming upright was used as the acute metric of injury. In an initial study, wild type mice (C57Bl6/J, Jackson Laboratory, Bar Harbor, ME) were randomly assigned to receive a single impact with piston pressure set to range between 0 and 2.4 psi. Injuries were performed using the same anesthesia and head placement protocol described above and righting time was recorded and used

to determine the subconcussive threshold. Mice injured with piston pressures greater than 1.9 psi (0.13J energy intensity) all had mean righting times greater than the sham average.

Previous work using the CHIMERA device has determined that a single impact below 0.5J does not result in phenotypic deficits, including any increases in LRR time.[29] However, our pilot study with a single impact showed that animals receiving impacts between energy intensities of 0.13J and 0.24J (3.1 psi piston pressure) had increased LRR times (Supplemental Figure 1A). Furthermore, because we were interested in the cumulative effect of repeated injuries rather than a single impact, a second study was performed using wild type mice. These mice were randomly assigned to receive 20x sham, 20x 0.13J or 20x 0.24J injuries spaced 24 ± 1 hour apart. Righting times were recorded daily. The average righting time over the course of the twenty injuries was then calculated for each group and analyzed using a one way ANOVA followed by a post-hoc Tukey test (Supplemental Figure 1B). There was a significant effect of injury in this study ($F_{(2,31)} = 28.99$, $\eta^2_p = 0.652$, $p < 0.00001$). Animals in the 0.24J group had increased righting times relative to shams and 20x 0.13J mice (20x sham vs 20x 0.24J, $p = 0.000126$, 20x 0.13J vs 0.24J, $p = 0.000125$). Based on these data we determined a subconcussive energy threshold of 0.13J and a concussive energy threshold of 0.24J.

Injury timeline

At four months of age, hTau mice were assigned to 20x concussive injury, 20x subconcussive injury, or 20x sham groups using a random number generator. Following impact or sham, each animal was placed supine in a warmed chamber (37°C) and monitored by an investigator blinded to injury status in a quiet, separate room until fully upright and ambulatory. LRR was recorded by this investigator. For sham animals, LRR was defined as the time from when isofluorane delivery was stopped to regaining consciousness. After regaining consciousness, each animal was returned to its home cage. This procedure was repeated for twenty consecutive days with injuries spaced 24 ± 1 hour apart, such that each animal received twenty head impacts or sham procedures (Figure 1A). A Cox regression for survival analysis was performed to determine the effect of injury on survival post injury (Figure 1B), and showed that there was a significant effect of injury status on survival ($p= 0.0304$), with a trend towards more deaths in the 20x concussive injury group.

Behavioral testing

All neurobehavioral testing was performed in a specified behavior room isolated from external noise and with light and sound controls. 60 dB white noise (Marpac Dohm-DS, Wilmington, NC, USA) and lighting (40 lux for Morris Water Maze, 20 lux for all other tests) was controlled for all testing. Neurobehavioral tests were performed during the workday (6am-6pm). All equipment was cleaned with 70% ethanol prior to each test to remove scents. Animals were allowed to acclimate in the room for at least one hour before testing. Following injuries, cages were renumbered such that the investigator remain to injury status during the following behavioral testing. Behavioral testing was performed at three time points, acutely, three months and one year following injuries. With the exception of tail suspension, all tests were recorded on video and subsequently analyzed using Panlab SMART (Harvard Bioscience, Holliston, MA, USA).

Social interaction and social novelty: Crawley's three chamber test was used to measure social interaction and social novelty.[30] Test mice were singly housed for 24 hours prior to testing. On testing day, the test mouse was placed in a 42 x 70cm three chamber box made of opaque white plastic. Following a five minute habituation session where the mouse was allowed to freely explore all three chambers, the test mouse was then confined to the middle chamber during preparation for the preference for sociability session. A stimulus mouse (male C57BL/6 aged 6-8 weeks) was placed in a wire cage in one chamber while a dummy mouse made of black Legos was placed in a wire cage in the opposite chamber. The test mouse was then allowed to explore all three chambers for ten minutes. The test mouse was then confined again to the middle chamber prior to preference for social novelty testing. The dummy mouse used during sociability testing was replaced with a novel stimulus mouse (male C57BL/6 aged 6-8 weeks). The test mouse was then allowed to explore all three chambers for ten minutes. Because each testing session required approximately 30 minutes of test time, stimulus mice were used in pairs and allowed to rest while a second pair of stimulus mice was used for the next test mouse. Time spent interacting with each mouse was measured by tracking the time the mouse spent sniffing within a 1.5cm radius around each wire cage. Activity such as climbing on top of the wire cage was not counted as interaction.

Elevated plus maze: A custom maze (30cm high) with arms that were 30cm long and 5cm wide was used for testing. Closed arms were enclosed by 16cm high walls while open arms had a 2mm railing to prevent mice from falling. Mice were placed in a closed arm and allowed to explore the maze for five minutes. A 5cm square

was defined as the center zone, requiring the mouse to have all four paws in an arm of the maze to be quantified in SMART as being in that arm. Time spent in all zones (open arms, closed arms, center zone) was quantified using SMART.

Open field test: Mice were placed in a 44.5 x 44.5cm box made of white opaque plastic and allowed to explore for five minutes. Thigmotactic behavior was defined as the average distance away from the wall. Briefly, the time spent moving was measured using Panlab SMART. During video analysis the box was divided into fifteen concentric zones spaced 1.5cm apart. Time spent in each zone was weighted by zone distance from the maze wall and summed to obtain a time-weighted average.

Morris Water Maze: A 120cm diameter pool was filled with 22°C water made opaque using non-toxic white tempera paint. For each day except for probe trial, mice were required to swim for four trials per day, and were inserted into four locations in the pool (NW, SW, SE, NE) and allowed to swim for a maximum of one minute to reach the 11 cm platform. The order of insertion points was changed each day. During the visible platform phase (three days, Wednesday-Friday), a flag was placed on the platform so that it would be clearly visible. For the hidden platform phase (four days, Monday-Thursday), opaque white curtains were hung to enclose the pool, with prominent visual cues in each quadrant. The platform was then moved to a different quadrant and the water level was increased so that the platform was hidden under 1cm of opaque white water. To control for memory retention between test sessions, the hidden platform was placed in a different quadrant for each test session. For hidden and visible platform testing, the average distance swum, average swim speed, and latency to platform were recorded and calculated using Panlab SMART. Twenty-four hours following the final day of hidden platform testing, the platform was removed from the pool. Mice were then subjected to a single probe trial, where each mouse was inserted at the insertion point furthest from where the hidden platform had previously been and allowed to swim for thirty seconds. The percentage of time spent in the target quadrant as well as mean proximity from the platform were recorded and calculated using Panlab SMART.

Tail Suspension: A white paper cone was placed on each animal's tail to prevent climbing behavior commonly exhibited in C57Bl/6 mice. Mice were then suspended from a rod at a height of 30cm by their tails. The total test time was six minutes. Time immobile, defined as any time the mouse was not moving except swinging from previous movement, was recorded manually by an investigator blinded to injury status.

Cerebrospinal Fluid, Blood, and Tissue collection

Following the one year time point of behavioral testing, mice were sacrificed. Animals were randomly assigned a new identification number to assure blinding during histological analysis. Mice were anesthetized with a 75 mg/kg dose of pentobarbital administered IP. When mice remained unresponsive to tail or toe pinch, they were placed on a pad heated to 37°C, and CSF was extracted from the cisterna magna.[31] Mice were then transcardially perfused using ice cold 0.3% PBS-heparin solution and approximately 800 microliters of blood was collected using the cardiac bleeding method. Following tissue extraction, the brain was separated into two hemispheres. The left hemisphere was drop fixed overnight in 4% paraformaldehyde and then switched to 30% sucrose solution, stored at 4°C. The right hemisphere was dissected on a chilled aluminum foil plate into cortical and hippocampal regions and stored at -80°C. Blood samples were allowed to clot for a minimum of one hour, and then blood and CSF were centrifuged at 2,000 x g to extract serum samples, which were stored at -80°C. Results from CSF, blood and frozen brain tissue analyses will be reported separately.

Immunohistochemistry

Fixed brain tissue was sectioned coronally into 50 µm thick sections using a freezing sliding microtome (Microm HM 430, ThermoFisher Scientific, Waltham, MA, USA) in a 1:6 series and stored in cryoprotectant at 4°C. Phosphorylated tau and activated astrocytes were detected using monoclonal AT8 (Cat# MN1020, ThermoFisher Scientific, Waltham, MA, USA) and polyclonal GFAP (Cat# AB5541, Millipore Sigma, St Louis, MO) respectively. For ptau staining, antigen retrieval was performed using a ten minute incubation in 70% formic acid. Sections were then incubated in 0.3% hydrogen peroxide solution to block endogenous peroxidase and then blocked for thirty minutes in 3% serum diluted in TBS-X. For AT8 staining normal goat serum (Cat# S-1000, Vector Laboratories, Burlingame, CA, USA) was used, while normal donkey serum (Cat# 017-000-121, Jackson Immunoresearch, West Grove, PA, USA) was used for GFAP staining. Sections were then incubated overnight at 4°C in primary antibody diluted 1:1000 in 3% serum. Tissue from an uninjured 22 month old 3xTG mouse was used as a positive control for ptau pathology, while tissue from a mouse injured using the controlled cortical impact injury model was used as a positive control for GFAP staining. Negative controls with the primary antibody excluded were performed for both stains. The next day, sections were incubated in secondary antibody diluted 1:1000 in TBS-X for one hour. For AT8 staining, biotinylated goat anti-mouse (Cat# BA-9200, Vector Laboratories, Burlingame, CA, USA) was used while biotinylated donkey anti-chicken (Cat# 703-065-115, Jackson Immunoresearch, West Grove, PA, USA) was used. Sections were incubated for one

hour in ABC solution (Cat# PK-6100, Vector Laboratories) diluted 1:400 A and B in 1xTBS, followed by visualization using 3'3' diaminobenzidine (Cat# D5905-100TAB, Sigma Aldrich, St. Louis, MO). Sections were washed three times in 1x TBS between each step.

Myelin Black Gold II staining

Tissue sections were first washed in TBS to remove residual cryoprotectant. Black Gold II solution was prepared by resuspending 150 mg dry Black Gold II powder (Cat# AG400, Millipore Sigma, St. Louis, MO) in 0.9% saline solution. The Black Gold II solution was then heated to 60°C. Free floating tissue sections were incubated in the solution for 8-12 minutes until fibers were completely stained (Supplemental Figure 2). Following two washes in Milli-Q water, sections were incubated in 0.1% sodium thiosulfate solution heated to 60°C for three minutes. The tissue sections were then washed three times in TBS to remove trace sodium thiosulfate.

Quantification of histology

Following staining, tissue sections were mounted onto positively charged slides, dehydrated in a series of graded ethanols (50%-70%-95%-100%-100%), and cleared in three changes of xylenes before being coverslipped (No. 1.5, Cat # 48393-241, VWR). Images of stained sections were acquired on a Zeiss Axioscan (Carl Zeiss AG, Oberkochen, Germany) using a brightfield 10x objective lens and then exported at their original resolution into tiff format for quantification. Regions of interest (ROIs) in the gray matter included cortical gray matter (4-5 sections/animal), lateral septal nucleus (1 section/animal), and the hippocampus (3-4 sections/animal). White matter intact (>50% of region without folds or tears) ROIs included the corpus callosum (4-5 sections/animal) anterior commissure (1 section/animal), hippocampal commissure (1 section/animal) and fimbria (2 sections/animal).

To quantify GFAP staining, the Renyi entropy thresholding method was applied to all images (Supplemental Figure 3). Similar to Myelin Black Gold II analysis, ROIs were included and considered intact only if >50% of the region was without folds or tears. To exclude remaining edge and fold artifacts, only particles with an area of 10-500 pixels were included in analysis.

Because there were no signs of severe demyelination in any of the tissue sections, we used power coherence as a measure of white matter integrity as previously described.[32] Briefly, each ROI was subdivided into square regions with an area of 10 pixels^2. This area was used in order to sustain sufficient spatial resolution and

prevent artifacts caused by multiple crossing fibers, while also removing noise related artifacts. A two tensor model was then fit to the power spectrum of each subdivided region. Power coherence, a metric ranging from zero to one was calculated by subtracting the ratio of the major and minor axes of the tensors from one.

PCR Confirmation of Genotype

PCR was used to confirm the hTau genotype from one animal selected at random from each cohort of hTau mice. DNA from the leftover regions of the fresh-frozen brain tissue was extracted using the EZ High MW Mouse Tail DNA Isolation Kit (Cat# M1007-100, EZ Bioresearch, St. Louis, MO). Nuclei lysis and Proteinase K solution were added to the tissue sample and incubated overnight at 55°C with gentle shaking until the tissue was completely digested. The next day, protein precipitation solution was added to the mixture. Following a five minute incubation on ice, the solution was centrifuged at 16,000 x g for three minutes at room temperature, separating the protein pellet from supernatant. Isopropanol was added to the supernatant and inverted until DNA was visible as threads. The supernatant and isopropanol solution was then centrifuged 16,000 x g for one minute at room temperature. The supernatant was then removed from the microcentrifuge tube and the remaining DNA pellet was washed with 70% ethanol. The DNA pellet was centrifuged and washed again with 70% ethanol. Any residual supernatant was then removed and the DNA pellet was allowed to air dry for ten minutes. DNA Hydration Solution was added to the pellet and incubated at 65°C for one hour with gentle shaking. The extracted DNA was then stored at 4°C until PCR genotyping. Tail DNA from an hTau mouse was obtained from an independent investigator to use as an external positive control.

Control (htau ctrl R : 5'- GTA GGT GGA AAT TCT AGC ATC ATC C -3', htau ctrl F : 5'- CTA GGC CAC AGA ATT GAA AGA TCT -3') and test oligomers (htau R : 5'- CTC TGC ATG GCT GTC CAC TAA CCT T -3', htau F : 5'- ACT TTG AAC CAG GAT GGC TGA GCC C -3') were resuspended to 100µM concentration in molecular grade water. The extracted DNA was thawed on ice and diluted to a final concentration of 100 ng/µL. A solution of DNA, control or hTau oligomer, GoTaq Flex, dNTP, reaction buffer and enhancer was then prepared in molecular grade water and heated sing a thermocycler. PCR was then performed using an agarose gel.

Statistical Methods

All analysis was performed using Statistica (Tibco Software Inc., Palo Alto, CA, USA). All data was tested for normality using the Shapiro-Wilk normality test. Any datasets which violated the assumption of normality were

transformed so that parametric analyses could be performed (Supplemental Table 2). A two-way, repeated measures ANOVA was performed for each neurobehavioral metric to test for the effects of injury and cohort, with test session (acute, three month, one year) being the within-subject factor. Two-way ANOVAs were used to analyze the LRR data and histological data. We prespecified the statistical plan to collapse across the two cohorts of mice for analyses for which there were no significant main effects or cohort of interactions with of other factors with cohort. Statistical power calculations for main effects of injury was 80% at $p<0.05$ with an effect size of 0.50. Animals that died before the end of the experiment were excluded from behavioral and histological analysis. Post-hoc Tukey tests were then used to determine significance for specific pairwise comparisons ($p<0.05$). Because the residuals were not normally distributed, a Spearman's rank correlation was used to determine the relationship between white matter power coherence and Morris water maze measures (four ROIs x five behavioral measures = 20 correlations). This was followed by Bonferroni correlation for multiple comparisons, where the effective number of tests was adjusted to account for potential correlation between measures.[33] Without this adjustment, the Bonferroni correction at $p<0.05/20 = 0.0025$, however, with the variance of the eigenvalues of the correlation matrix ($v_{\lambda obs} = 1.265$), the adjusted Bonferroni at $p <0.05/18.79 = 0.0027$. Effect sizes were calculated for each test that was performed (Supplemental Table 3). Graphs were plotted using Prism v7.0 (Graph Pad, La Jolla, CA, USA). All error bars represent standard error of mean (SEM).

Results

hTau mice show injury dependent differences in righting time

Loss of consciousness, measured by righting time (LRR) was used to measure the immediate effects of injury during the twenty days of impacts (Figure 2). LRR was calculated by measuring the righting time each day of injury, and averaging across days. A two way ANOVA showed that there was a significant effect of injury ($F_{(2,44)} = 11.8092$, $\eta^2_p = 0.349$, $p < 0.0001$). Animals in the 20x concussive group had elevated righting times compared to 20x shams ($p = 0.00059$) as well as animals in the 20x subconcussive group ($p = 0.00046$) after post hoc Tukey testing. There was no difference in righting times between 20x shams and animals in the 20x subconcussive group ($p = 0.985$). There was no effect of cohort or interaction between injury and cohort. These data show that the higher energy concussive impacts caused a significantly more substantial acute injury than the lower energy subconcussive impacts.

hTau mice subjected to repeated concussive injuries show persistent chronic cognitive deficits

At acute, three month and twelve month time points, animals were tested for learning deficits using the Morris Water Maze task. During the three days of visible platform testing (Figure 3, Supplemental Figure 4), the two way, repeated measures ANOVA, showed a main effect of injury on swim speed ($F_{(2,36)} = 29.9335$, $\eta^2_p = 0.624$, $p < 0.00001$). Animals in the 20x concussive group had reduced swim speeds relative to both shams ($p = 0.000127$) and mice in the 20x subconcussive injury group ($p = 0.000128$). There was no interaction between test session (acute, three month, twelve month) and injury. There was also a main effect of injury on swimming distance ($F_{(2,36)} = 25.642$, $\eta^2_p = 0.588$, $p < 0.00001$). Animals in the 20x concussive group had increased swimming distances relative to shams ($p = 0.000127$) and mice in the 20x subconcussive injury group ($p = 0.000127$). There were no differences between subconcussive injury and sham groups ($p = 0.923$). There was no effect of test session or interaction between test session and injury on swim speed or distance swum.

At each test session, two days following the final day of visible platform testing, animals underwent hidden platform testing of the Morris Water Maze task for four consecutive days (Figure 4, Supplemental Figure 5). Similar to the visible platform phase, the two way repeated measures ANOVA showed an effect of injury on swim speed ($F_{(2,36)} = 36.5009$, $\eta^2_p = 0.663$, $p < 0.00001$) that remained significant after post-hoc testing. Mice in the 20x concussive group had reduced swim speeds relative to 20x shams ($p = 0.000125$) and mice in the 20x subconcussive group ($p = 0.000128$). Animals in the in the 20x subconcussive group also had reduced swimming speeds relative to 20x shams ($p = 0.028$). There was also an effect of injury on swimming distance during the hidden platform phase ($F_{(2,36)} = 6.272$, $\eta^2_p = 0.564$, $p<0.01$) that remained significant following post-hoc Tukey testing. Animals in the 20x concussive group had increased swimming distances relative to 20x shams ($p = 0.044$) and animals in the 20x subconcussive group ($p = 0.009$). There was no difference in swimming distance between subconcussive injury and sham groups ($p = 0.867$). There was no effect of test session on either swim speed or swimming distance. There was also no interaction between test session and injury on either of these measures.

Twenty four hours after the final day of hidden platform testing, hTau mice were tested for memory retention using the probe trial of the Morris Water Maze task (Figure 5, Supplemental Figure 6). Mean proximity to platform and percent time spent in the target quadrant were used as metrics of memory retention for each test session. For the measure of mean proximity to platform, there was an effect of injury ($F_{(2,36)} =$

23.173, $\eta^2_p = 0.570$, $p < 0.00001$) and time ($F_{(2,70)} = 6.9935$, $\eta^2_p = 0.167$, $p < 0.01$) on mean proximity to platform that remained significant after post-hoc testing. Animals in the 20x concussive injury group were further away from where the hidden platform had previously been placed relative to both shams ($p = 0.000124$) and 20x subconcussive animals ($p = 0.000126$). Animals also had increased proximity to the target platform during the three month test session ($p = 0.000280$) relative to the acute test session. There was also an effect of injury on percent time in the target quadrant ($F_{(2,36)} = 22.851$, $\eta^2_p = 0.566$, $p <0.00001$) that remained significant after post-hoc testing. These same animals also spent less significantly less time in the target quadrant relative to shams ($p = 0.000124$) and 20x subconcussive animals ($p = 0.000123$). There were no significant differences between animals in the 20x subconcussive group or 20x sham group for either proximity to platform or percent time spent in the target quadrant. There was no interaction between time and injury for either parameter.

In summary, repetitive concussive injury resulted in reduced swim speed during both visible and hidden platform phases of Morris Water Maze, a possible indication of reduced motor function or motivation following injury. Furthermore, repetitive concussive injury also caused an increase in distance swum to the target. This effect indicates an impairment in ability to learn the platform location. Moreover, repetitive concussive injury resulted in impaired memory, demonstrated by increased proximity to platform and reduced percent time in target quadrant during the probe trial. These findings, combined with the effect of time on proximity to the platform during only intermediate session of probe trial indicates that the 20x concussive injury method implemented in this study causes acute cognitive deficits that neither progressively worsen nor resolve at one year post injury. Instead, these changes in learning and memory appear to remain sustained at a reduced level relative to shams and animals in the 20x subconcussive group.

No social interaction deficits, anxiety or depressive-like behaviors detected after repeated injury in hTau mice

The sociability and social novelty phases of Crawley's three chamber social interaction test were used to measure both acute and chronic social behaviors (Figure 6, Supplemental Figure 7). For the sociability test, the ratio of time spent interacting with the stimulus mouse to time spent interacting with the novel object was used as an index of sociability. For the social novelty test, the ratio of time spent interacting with the novel mouse to time spent interacting with the previous stimulus mouse was used as an index of social novelty. The

two way repeated measures ANOVA showed no effect of injury on either sociability index ($F_{(2,37)}$ = 0.300, p = 0.74) or social novelty testing index ($F_{(2,37)}$ = 2.729, p = 0.78). There was also no effect of test session on either index.

Anxiety-like behavior was measured using two behavioral measures, open arm time during the elevated plus maze task, and thigmotaxis during the open field maze task (Figure 7, Supplemental Figure 8). Animals displaying increased risk taking and anxious behavior would be expected to have increased open arm time and elevated thigmotaxis scores. There was no effect of injury on open arm time ($F_{(2,37)}$ = 0.195, p = 0.98). While the two way repeated measures ANOVA did show an effect of injury on open field thigmotaxis ($F_{(2,37)}$ = 3.610, p = 0.0367) there were no significant differences between groups following post-hoc testing. There was no effect of test session on open arm time or thigmotaxis.

Time immobile during the tail suspension test was used as a metric of depressive-like behavior (Figure 8, Supplemental Figure 9). The two way repeated measures ANOVA showed an effect of injury on immobility time ($F_{(2,37)}$ = 12.954, p < 0.0001) that remained significant after post-hoc testing. hTau mice in the 20x concussion group showed reduced immobility times rather than the expected increased immobility times relative to shams (p = 0.00427) and relative to mice in the subconcussive group (p = 0.000212). The interpretation of this result is not clear (see discussion). There was no significant difference between animals in the 20x subconcussive group and 20x shams (p = 0.546). There was also no significant effect of test session on time spent immobile, though it appeared that the effect was less apparent at the 3 month test session. These findings show that repetitive concussive or subconcussive injury does not result in acute social, anxiety related, or depressive-like deficits. Furthermore, based on the lack of an effect of test session, deficits in these domains do not manifest during the chronic stages following injury.

Repetitive brain injury results in localized chronic astrogliosis

GFAP staining was used to assess the effect of repetitive head injury on chronic astrogliosis. Regions of interest were drawn in the gray matter (cortex, lateral septal nucleus and hippocampus) and white matter (corpus callosum, anterior commissure, hippocampal commissure and fimbria). Percent area of positive staining was used as a measure of astrogliosis. The two way ANOVA showed no effect of injury on percent area of GFAP staining in any of the regions of interest (Figure 9). In summary, our analysis of activated astrocytes show that our 20x concussion model does not result in chronic astrogliosis.

No evidence of phosphorylated tau pathology in hTau mice

PCR genotyping confirmed that mice from both cohorts were positive for human tau (Supplemental Figure 10). However, hTau mice did not show any substantive staining for HT7 (total tau), AT8 (ptau, ser202/thr205), CP13 (ptau, ser202) or RZ3 (ptau, thr231) (Figure 10A). A 22 month old 3xTG mouse, used as a positive control for total tau and ptau staining, showed extensive positive staining for total tau and phosphorylated tau epitopes (Figure 10B).

Repetitive injury results in chronic white matter disruption

Power coherence analysis of Myelin Black Gold II stained images was used to measure chronic white matter disruption (Figure 11). White matter regions of interest included the corpus callosum, anterior commissure, hippocampal commissure and fimbria. The two way ANOVA showed an effect of injury on power coherence in the corpus callosum ($F_{(2,37)} = 28.048$, $\eta^2_p = 0.644$, $p < 0.00001$), hippocampal commissure ($F_{(2,37)} = 34.015$, $\eta^2_p = 0.687$, $p < 0.00001$) and fimbria ($F_{(2,37)} = 17.262$, $\eta^2_p = 0.527$, $p < 0.00001$) that remained significant following post hoc Tukey testing (Figure 12). hTau animals in the 20x concussive group had reduced power coherence in the corpus callosum ($p = 0.000125$), hippocampal commissure ($p = 0.000125$) and fimbria ($p = 0.000136$) relative to shams. Animals in the 20x subconcussive group also showed more modestly reduced power coherence only in the corpus callosum ($p = 0.0091$) and hippocampal commissure ($p = 0.027$) relative to shams. Power coherence was also significantly reduced in the corpus callosum ($p = 0.000367$), hippocampal commissure ($p = 0.000150$), and fimbria ($p = 0.000189$) in the 20x concussive group relative to animals in the 20x subconcussive group. The injury dependent reduction in power coherence suggests that repetitive brain injuries cause acute white matter disruption that remains unresolved in the chronic phase of injury. The difference in effect sizes when comparing power coherence in white matter indicates that the corpus callosum and hippocampal commissure may be especially useful regions to assess chronic histological outcomes.

Correlation between white matter disruption and behavioral performance deficits.

To address the question of whether there was a relationship between white matter disruption and the cognitive and motor deficits observed during the Morris water maze task, we performed Spearman's rank correlations between white matter power coherence data collected from the chronic test session of Morris water maze (Figure 13). This was followed by Bonferroni correction for multiple comparisons where the effective number of

tests was adjusted by the variance of the eigenvalues of the correlation matrix.[33] We found a modest but significant relationship between power coherence in the corpus callosum and swimming speed to the target platform during both visible and hidden phases. When analyzing the 20x concussive group, this correlation remained moderate and significant. There was also a moderate relationship between white matter power coherence in the hippocampal commissure and swimming speed to the target platform during both visible and hidden phases. However, when considering the 20x concussive injury group, this relationship was weak and not significant. Finally, we found a modest but significant relationship between power coherence in the fimbria and proximity to platform during probe trial. This relationship weakened and was not significant when considering the 20x concussive group alone. These results indicate that white matter damage in the corpus callosum, hippocampal commissure, and fimbria is related to behavioral deficits exhibited during the Morris water maze.

Discussion

We have implemented a repetitive subconcussive and concussive brain injury paradigm and reported acute, subacute, and chronic behavioral outcomes, as well as histological outcomes at one year following injury. We found that repetitive concussive injuries in adult mice result in acute cognitive and motor dysfunction that remains unresolved at up to one year post injury. Furthermore, repetitive concussive injuries caused chronic white matter disruption in several regions of the brain that was moderately correlated with deficits in swimming speed and memory during Morris water maze testing.

Our findings show some consistencies with previous repetitive brain injury models. Multiple repetitive injury models have shown that daily injuries result in cognitive deficits that manifest acutely and persist as late as one year following injury.[8, 9, 21, 34, 35] These results show some relevance to the manifestation of behavioral symptoms in patients diagnosed with CTE post-mortem. These patients can be classified into two categories: the first group of patients present with mood and behavioral disturbances at an early age (~ 35 years old) followed by later cognitive deterioration, while the second group presents with cognitive symptoms at an older age (~60 years old) followed by further progression into dementia.[36] The robust findings of persistent cognitive impairment are most clearly relevant to the second group. Preliminary data has shown that patients who were diagnosed with CTE and had increased cognitive reserve show delayed onset of behavioral symptoms,

indicating a potentially important role of cognitive reserve in regulation of mood and anxiety related behaviors.[37]

When studying other behavioral domains, such as anxiety, social, or depressive behavior, the results become more challenging to reproduce in animal models. For example, mice injured using repetitive injury paradigms do not consistently show deficits when assayed using the open field maze or elevated plus maze, a result paralleled in our work. This could be explained by the sensitivity of these tests to external conditions, such as background noise, lighting conditions and time of day. In particular, tests such as open field maze lack a standardized metric, instead often using distance traveled or time in a user defined periphery zone of the testing box. We attempted to address this deficiency by using time-weighted distance from the maze center as a measure of thigmotaxis, but still found no differences in anxiety related behavior following injury. Our findings in relation to depressive behavior using the tail suspension test proved to be counterintuitive. Although injured animals showed impaired swimming speed during Morris water maze, injured animals had greater mobility times during the tail suspension task, a finding contradicted by previous work using the same behavioral assay.[6, 38] The increased mobility may indicate increased hyperactivity following injury. However, sham hTau animals also had surprisingly long immobility times compared to wild type animals of the same age. [39] The findings are therefore difficult to interpret without additional data from other tests of depressive behavior such as sucrose preference or the forced swim test, as well as testing tail suspension in the absence of prior stressors such as Morris water maze testing. [40, 41]

Interpretation of our histological findings in the context of previous work shows mixed results. Our data show injury related differences in white matter integrity measured using power coherence. Furthermore, there were region specific correlations of white matter disruption and impairments in swimming speed and spatial memory, specifically in the corpus callosum, hippocampal commissure, and fimbria. The large effect sizes in both the corpus callosum and hippocampal commissure indicate that these regions may be useful targets when assessing chronic histological outcomes following therapeutic trials. Previous repetitive injury paradigms have shown reduced white matter volume in the intermediate period following injury [11] but detected no changes in SMI31 immunoreactivity at one year post injury. [34] We have previously observed reduced white matter integrity measured by power coherence in the regions adjacent to tau laden sulcal depths in ex vivo Stage III/Stage IV CTE human cortical tissue. [42] However, it remains uncertain whether this white matter disruption is

a consequence of acute axonal injury, myelin degeneration, or an increased microglial or astrocytic response.[21] Furthermore, our findings of correlations of fiber integrity in the corpus callosum and hippocampal commissure with swim speed were surprising and indicate a need for further investigation into the microstructural changes in gray matter in the chronic phase following brain injury, which may be better predictors of cognitive outcomes such as swimming distance. Our attempt to address this question by measuring chronic astrogliosis showed no differences in GFAP immunoreactivity between sham and injured groups. We used percent area of GFAP staining as our metric, which is not sensitive to morphological differences in astrocytes. Quantification of changes in astrocyte process and cell body shape would be an alternative method, but would require the use of fluorescent markers and image acquisition at high magnifications for optimal analysis.[43] However, with the growing interest in the relationship between astrocytes and ptau in neurodegenerative disease, such experiments may be worth exploring.[44] Additionally, because microgliosis may have an important role in many tauopathies including CTE, exploration of microglial morphology using CD68 or Iba1 staining could provide further insight into the sequence of neurodegeneration.[45] A further area requiring future investigations will be analysis of synapse loss and its relationship with behavioral deficits.

The main strength of this study is that it is one of a few that has followed a population of mice out to 16 months of age and recorded behavioral data from the same mice at acute, intermediate (three month) and chronic (one year) time points post injury. Importantly, the injury related deficits in learning in memory we observed are apparent at the chronic time point, while previous studies have shown cognitive and behavioral deficits acutely that either resolve by the intermediate phase, or do not use a more long term time point (9-12 months post injury) when measuring chronic behavioral outcomes. There are also several strengths when considering the design on the study. First, the use of the transgenic hTau mouse line allowed us to eliminate potential confounds of the differences in tau isoforms between mice and humans. Second, mice were injured at four months of age, when phosphorylated tau tangles are not yet present in the hippocampus or cortex. This age at time of injury selected to better parallel the age at which many patients with CTE sustain repetitive injuries.[46] Third, our choice of the non-surgical CHIMERA model allowed for increased number and frequency of injuries that combined both direct impact and rotational acceleration components experienced during a traumatic brain injury.

The negative outcomes of this study reveal several limitations. The first and perhaps most important, is the choice of energy level for repeated concussive and subconcussive impacts. Our LRR data used to inform the selection of 0.13J and 0.24J for subconcussive and concussive injuries showed that animals injured with at least one impact had increased righting times relative to shams. Moreover, we found that animals injured twenty times with 0.24J impacts had increased righting times relative to animals in the 20x subconcussive group and shams, as well as a significantly reduced survival rate in the intermediate and chronic phases following injury. These findings are inconsistent with previous studies using the CHIMERA device, which defined injuries with energy levels below 0.4J as “subthreshold”.[29] However, these assessments were performed after a maximum of two impacts and did not address the question of cumulative effects of repetitive subthreshold injuries.[29, 47] Previous work in the field of repetitive subthreshold injuries in a rat model has shown that while a single subthreshold impact does not result in pathology, repeated injuries can acutely result in histological abnormalities such as increases in microtubule associated protein, phosphorylated neurofilament, and increased pTau immunoreactivity.[48] In humans, a combination of number of injuries as well as impact velocity shows a threshold-dose response when predicting late-life cognitive dysfunction and mood dysregulation.[49] One possible future direction would be to increase the energy intensities, using 0.4J as the concussive threshold while maintaining the same frequency and number of injuries. This might better address the question of the differences in neurobehavioral and neuropathological trajectory following repetitive subconcussive and concussive injuries.

A second limitation is the use of a lissencephalic animal when attempting to develop an animal model of CTE, where the formation and distribution of ptau pathology is attributed to injury of cortical tissue. In humans, these structures are highly folded, and the curvature of fibers may result in increased vulnerability to mechanical insult and subsequent aggregation of ptau tangles and astroglial tau.[50] Additionally, the composition of the rodent brain, which has a substantially lower white:gray matter ratio relative to humans could also be a reason for the lack of replication of ptau pathology found in CTE.[51] Future studies in animals with gyrencephalic brain architecture, such as ferrets or pigs, would be more relevant when understanding the relationship between repetitive brain injury and chronic histopathological and behavioral deficits found in humans.[26, 52-54]

An unexpected limitation in this work is the lack of tau pathology in the hTau mice. Previous studies in the hTau mouse line have shown that uninjured animals are expected to develop neurofibrillary tangles in the hippocampus and cortex starting at nine months of age, verified using immunohistochemistry, Western blot, and ELISA assays.[18, 19, 55] PCR confirmed the hTau genotype of the two cohorts of mice used in this study. However, an array of monoclonal antibodies specific for both total tau and ptau showed minimal staining in hTau mice compared to a 22 month old 3xTG positive control. While the positive control would be expected to have increased tau staining because of the triple transgene, this does not explain the lack of positive staining in the hTau line.[56] Instead, this could be attributed to genetic drift in the mouse line. The choice of pentobarbital as anesthesia during CSF collection and tissue extraction is also a potential confound of this study. The reduction in respiratory rate during this mode of anesthesia could result in unexpected histopathological effects, such as an altered inflammatory or astrocytic response.[43, 57] While we did not see any differences in astrocytosis as evidenced by GFAP staining in any region, an acute astroglial response following pentobarbital injection would explain the levels of astrocytes observed in the gray and white matter regions of both injured and sham hTau mice. This would be best addressed by performing GFAP staining in animals perfused using an alternative method of anesthesia, such as isofluorane, on littermates.

Additional future directions of this study include analysis of the CSF and blood samples for tau, GFAP, and other brain proteins. These will allow us to explore the question of whether repetitive brain injury results in changes of soluble tau species or impairs long-term blood brain barrier integrity. Furthermore, ELISAs and other assays of the fresh-frozen tissue samples collected from the hTau mice will provide an alternative to histopathology to determine whether there is evidence of phosphorylated tau in the brain.

In summary, a repetitive CHIMERA injury paradigm implemented in adult mice results in persistent behavioral dysfunction that is linked to disruption of specific white matter tracts. This disruption, detected by calculation of power coherence from myelin staining, shows that white matter damage occurs in a graded manner based on the intensity of repeated impacts, coinciding with behavioral deficits. These outcomes show that impacts previously considered “subthreshold” do result in lasting tissue damage in the brain.

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Tables

Behavioral measure	White matter region	$p_{full\ sample}$	$r_{full\ sample}$	$P_{20x\ Sham}$	$r_{20x\ Sham}$	$p_{20x\ Subconcussive}$	$r_{20x\ Subconcussive}$	$p_{20x\ Concussive}$	$r_{20x\ Concussive}$
Visible swim speed	Corpus Callosum	0.000222	0.578	0.1930	0.312	0.1822	0.396	0.0234	0.721
Hidden swim speed	Corpus Callosum	0.002689	0.485	0.1014	-0.478	0.5053	0.203	0.0234	0.721
Visible swim speed	Hippocampal Commissure	0.00262	0.493	0.1460	-0.429	0.4169	0.259	0.9184	0.0424
Hidden swim speed	Hippocampal Commissure	0.000409	0.558	0.6300	-.1484	0.1474	0.4476	0.9673	0.0182
Probe trial: proximity to platform	Fimbria	0.00228	-0.499	0.4477	-0.2308	0.9910	-.00700	0.4069	-0.297

Table 1. Correlations of Morris water maze behavior and white matter integrity. A Spearman's correlation followed by adjusted Bonferroni correction for multiple comparisons was used to determine whether there were any significant correlations between white matter power coherence and the cognitive and motor dysfunctions observed during Morris water maze.

Figure Legends

Figure 1. Injury timeline and survival post injury. A. Fifty male hTau mice were purchased in two separate cohorts. Animals were randomly assigned to sham, 20x subconcussive (0.13J) or 20x concussive (0.24J) injury groups. Following twenty days of injuries, behavioral outcomes including social interaction (SI), elevated plus maze (EPM), open field maze (OFM), Morris water maze (MWM) and tail suspension (TS) were assessed acutely, at three months, and one year post injury. Following the 12 month behavioral test session, all animals were perfused. **B.** Survival and mortality risk were assessed using a Cox survival and hazard regression analysis. The data showed a modest but significant (p=0.03) effect of subconcussive or concussive injury on mortality rate.

Figure 2. Loss of consciousness following injury. Latency righting reflex was used as to measure loss of consciousness. Righting times were measured by an observer blinded to injury status. Animals in the concussive injury group had increased righting times relative to the subconcussive and sham groups, which were indistinguishable. There was a significant effect of concussive injury on righting time, with animals in the 20x concussive group having increased righting times relative to 20x shams (p = 0.00059) and 20x subconcussive mice (p = 0.00046).

Figure 3. Effects of injury on visible platform phase of the Morris water maze. A. Average swimming speeds for each day of the visible phase of the Morris water maze during each of the three test sessions. There was a significant effect of injury on swim speed (0.24J vs sham: p = 0.000127, 20x concussive vs 20x

subconcussive: p = 0.000128). There was no interaction between test session and injury. **B.** Total distance to the target platform during three days of visible platform testing. There was a significant effect of repetitive concussive injury (20x concussive vs sham: p = 0.000127, 20x concussive vs 20x subconcussive: p = 0.000127).

Figure 4. Effects of injury on hidden platform phase of the Morris water maze. A. Average swimming speeds for each of the four days of the hidden platform task during each of the three test sessions. There was a significant effect of injury (20x concussive vs sham, p = 0.000125, 20x concussive vs subconcussive, p = 0.000128). Mice in the 20x subconcussive group also had reduced swimming speeds relative to shams (p = 0.028). **B.** Total distance to the target platform during four days of hidden platform testing. There was a significant effect of concussive injury (20x concussive vs sham: p = 0.044, 20x concussive vs 20x subconcussive: p = 0.009). There was no interaction of test session and injury.

Figure 5. Effects of injury on Morris water maze probe trial performance. A. There was a significant effect of concussive injury on average proximity to platform during probe trial; 20x concussive vs 20x sham: p =0.000124, 20x concussive vs 20x subconcussive: p = 0.000126). There was also an effect of time, where animals were significantly further away from the platform at the 3 month test session (p = 0.000280) relative to the acute session (###). Animals in the repetitive concussive injury group were on average further away from the location of the target platform. **B.** There was a significant effect of concussive injury on the percent time spent in the quadrant where the platform had previously been placed (20x concussive vs sham: p =0.000124, 20x concussive vs 20x subconcussive: p = 0.000123). Animals in the repetitive concussive injury group tended to spend less than 25% of their time (chance performance, dotted line) in the target quadrant. There was no interaction between test session and injury.

Figure 6. No change in social interaction following injury. A. Injured animals did not show any significant changes in sociability compared to shams. Dotted line indicates a sociability index of one. Points above this line indicate that the animal showed preference for the stimulus mouse rather than the novel inanimate object. **B.** Injured animals did not show any significant changes in social novelty compared to shams. Dotted line indicates a social novelty index of one. Points above this line indicate that the animal showed preference for the novel mouse rather than the stimulus mouse from the previous sociability session.

Figure 7. No changes in anxiety related behavior following injury. A. Animals in the 20x concussive and 20x subconcussive did not show any changes in percent time spent in open arms of the elevated plus maze relative to shams. **B.** Injured animals did not show any changes in open field thigmotaxis (time weighted distance from the wall) relative to sham animals.

Figure 8. Effects of injury on depressive-type behavior. The tail suspension test was used to assess depressive-type behavior in animals. There was a significant effect of injury (20x concussive vs sham: $p = 0.00427$, 20x concussive vs 20x subconcussive: $p = 0.000212$) with less immobility in the 20x concussive group.

Figure 9. No effects of injury on chronic astrogliosis. A. Percent area of GFAP staining was measured in cortical gray matter. There were no differences in staining between injury groups. **B.** There was no difference in GFAP staining between injury groups in the lateral septal nucleus. **C.** No differences in GFAP staining were detected in the hippocampus **D.** No differences in GFAP staining were detected in the anterior commissure. **E.** There were no differences between injury groups detected in the hippocampal commissure. **F.** No differences in GFAP staining were detected in the fimbria. **H.** GFAP staining in a positive control wild type mice injured using CCI shows extensive astrogliosis in the hippocampus contralateral to the injury site. Scale bar = 100 microns.

Figure 10. No detection of tau pathology using immunohistochemistry in any of the hTau mice. A. Monoclonal AT8 (ptau, ser202/thr205), CP13 (ptau, ser202), RZ3 (ptau, Thr231) and HT7 (total tau) were tested to detect phosphorylated and total tau in the hTau mice line. No positive staining was observed for any of the antibodies in any of the groups. **B.** Tissue from a 22 month old 3xTG mouse was used as a positive control. When stained with AT8, CP13, RZ3 and HT7 there is extensive positive staining in the hippocampal neurons. Scale bar = 100 microns.

Figure 11. Effects of injury on chronic white matter disruption, assessed using power coherence. A. Power coherence was measured in the corpus callosum of tissue sections stained with Myelin Black Gold II. Fibers in sham animals have high coherence, indicating that fibers are aligned and intact. However, white matter fibers in injured animals show reduced power coherence, indicating white matter disruption. **B.** Fibers in the anterior commissure do not show any differences in power coherence, indicating intact white matter. **C.** Similar to the corpus callosum, white matter in the hippocampal commissure has reduced power coherence,

indicating fiber disruption. **D.** White matter in the fimbria of sham and 20x subconcussive animals shows high power coherence, while the fimbria of 20x concussive animals shows reduced power coherence. Because the fimbria had a tendency to stain more heavily relative to other regions, brightness and contrast levels were uniformly adjusted adjusted for the exemplars so fibers could be better visualized. Scale bars = 50 microns.

Figure 12. Reduced white matter integrity following repetitive injury. A. Power coherence in the corpus callosum was significantly reduced in injured animals (20x concussive vs sham, p = 0.000125, 20x subconcussive vs sham, p = 0.0091). There was also a significant difference in power coherence between animals in the 20x concussive and 20x subconcussive groups (p = 0.000367). **B.** Power coherence in the anterior commissure showed no effect of injury. **C.** There was a significant effect of injury on power coherence in the hippocampal commissure of hTau animals (20x concussive vs sham, p = 0.000125, 20x subconcussive vs sham, p = 0.027). There was also a significant difference between animals in the 20x concussive and 20x subconcussive groups (p = 0.000150). **D.** Power coherence in the fimbria was significantly reduced in animals in the 20x concussive group (20x concussive vs sham, p= 0.000136, 20x concussive vs. 20x subconcussive, p = 0.000189).

Figure 13. Relationship between white matter disruption and Morris water maze deficits. A. There was a moderate but significant relationship between power coherence in the corpus callosum and swimming speed during the visible platform phase in animals sustaining repetitive concussive injuries. This relationship was weak but significant in the hippocampus. **B.** A similar trend was observed in both the corpus callosum and hippocampal commissure during the hidden platform phase. **C.** A negative, weak but significant relationship between power coherence in the fimbria and proximity to platform during probe trial. Animals that were further away from the platform had a tendency to have sustained more severe chronic white matter damage in the fimbria.

Figure 1.

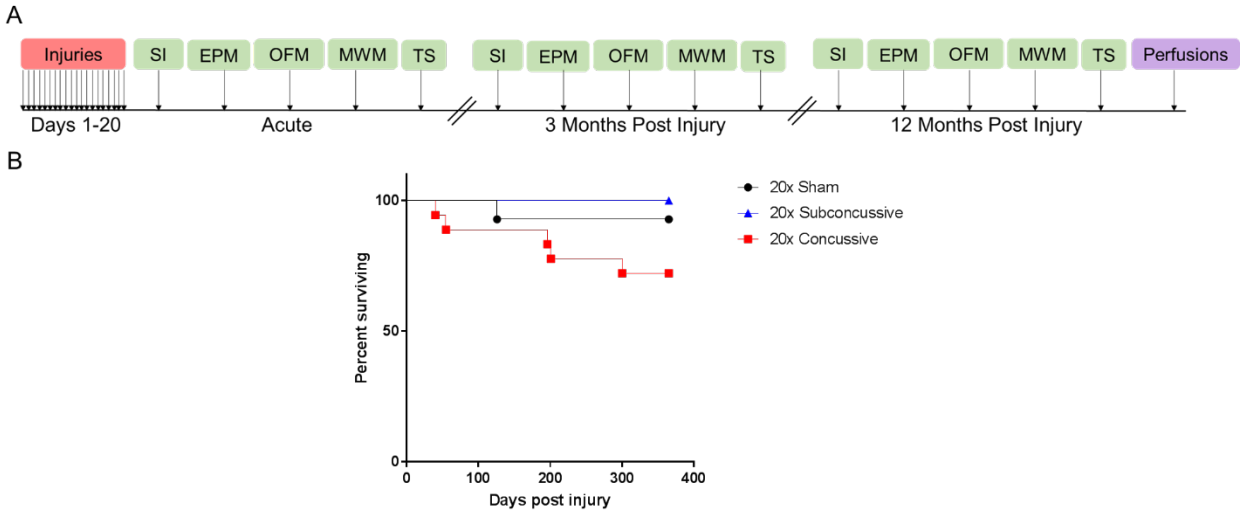


Figure 1. Injury timeline and survival post injury. A. Fifty male hTau mice were purchased in two separate cohorts. Animals were randomly assigned to sham, 20x subconcussive (0.13J) or 20x concussive (0.24J) injury groups. Following twenty days of injuries, behavioral outcomes including social interaction (SI), elevated plus maze (EPM), open field maze (OFM), Morris water maze (MWM) and tail suspension (TS) were assessed acutely, at three months, and one year post injury. Following the 12 month behavioral test session, all animals were perfused. **B.** Survival and mortality risk were assessed using a Cox survival and hazard regression analysis. The data showed a modest but significant ($p=0.03$) effect of subconcussive or concussive injury on mortality rate.

Figure 2.

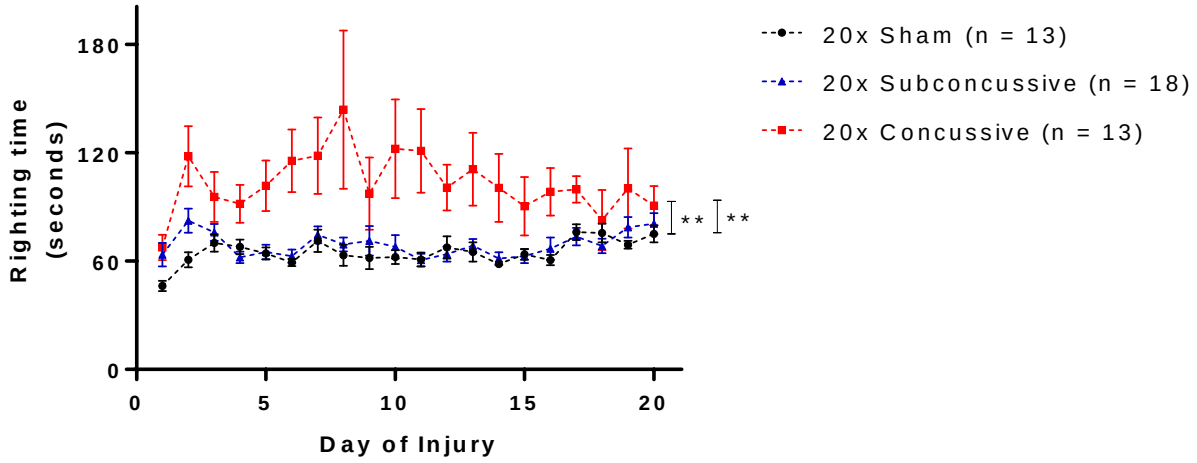


Figure 2. Loss of consciousness following injury. Latency righting reflex was used as to measure loss of consciousness. Righting times were measured by an observer blinded to injury status. Animals in the concussive injury group had increased righting times relative to the subconcussive and sham groups, which were indistinguishable. There was a significant effect of concussive injury on righting time, with animals in the 20x concussive group having increased righting times relative to 20x shams ($p = 0.00059$) and 20x subconcussive mice ($p = 0.00046$).

Figure 3.

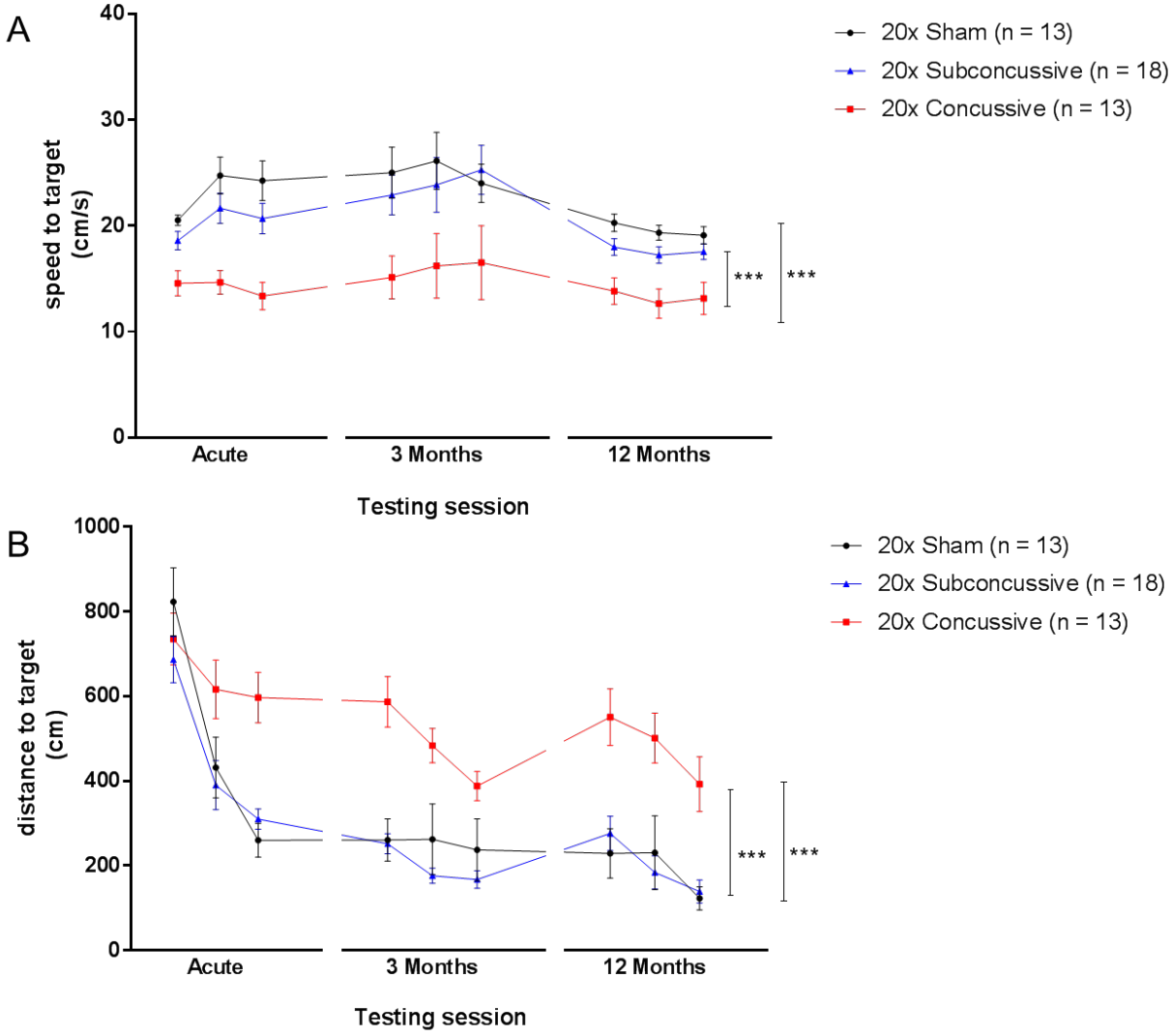


Figure 3. Effects of injury on visible platform phase of the Morris water maze. A. Average swimming speeds for each day of the visible phase of the Morris water maze during each of the three test sessions. There was a significant effect of injury on swim speed (0.24J vs sham: p = 0.000127, 20x concussive vs 20x subconcussive: p = 0.000128). There was no interaction between test session and injury. **B.** Total distance to the target platform during three days of

visible platform testing. There was a significant effect of repetitive concussive injury (20x concussive vs sham: p = 0.000127, 20x concussive vs 20x subconcussive: p = 0.000127).

Figure 4.

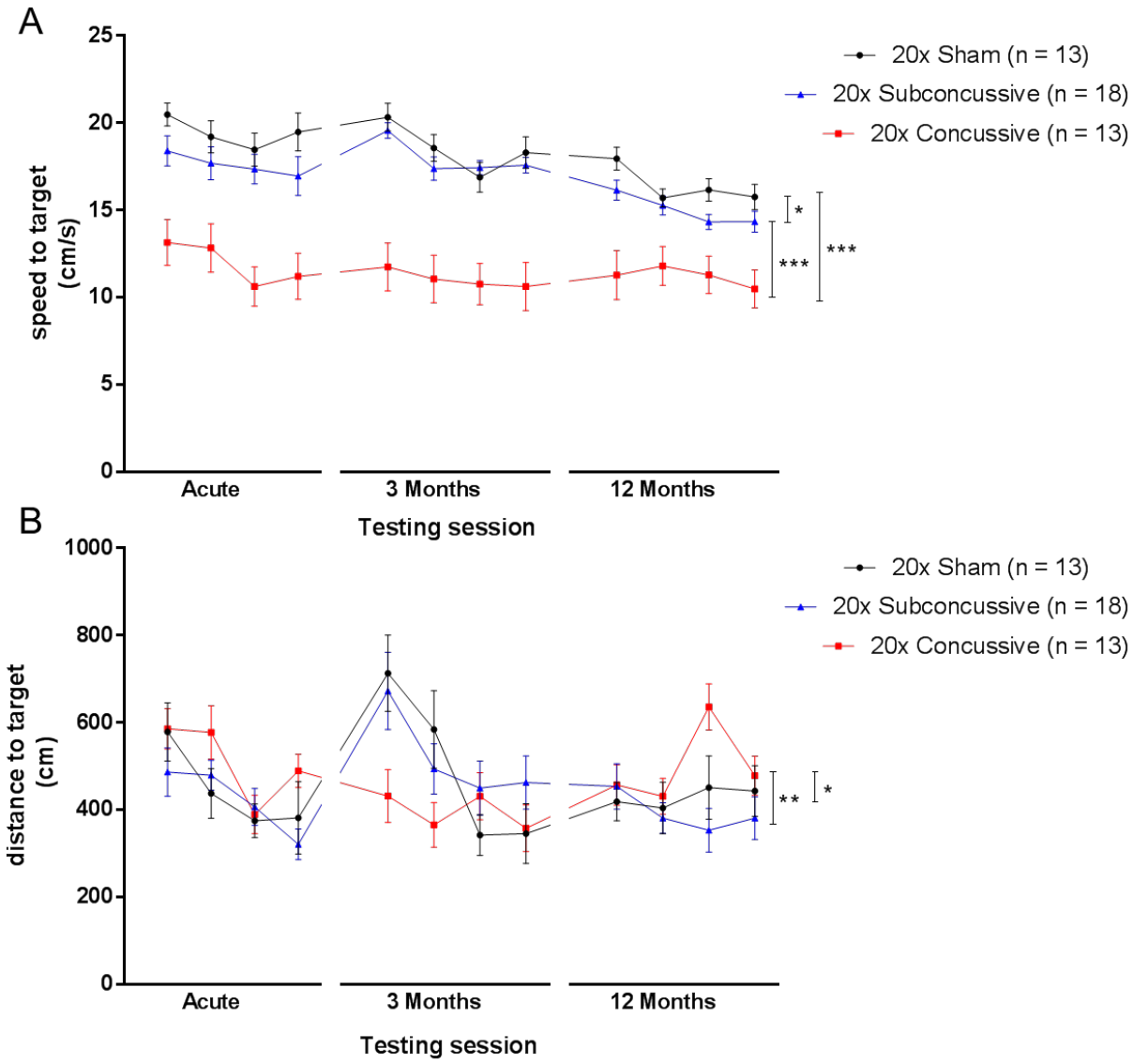


Figure 4. Effects of injury on hidden platform phase of the Morris water maze. A. Average swimming speeds for each of the four days of the hidden platform task during each of the three test sessions. There was a significant effect of injury (20x concussive vs sham, p = 0.000125, 20x concussive vs subconcussive, p = 0.000128). Mice in the 20x subconcussive group also

had reduced swimming speeds relative to shams (p = 0.028). **B.** Total distance to the target platform during four days of hidden platform testing. There was a significant effect of concussive injury (20x concussive vs sham: p = 0.044, 20x concussive vs 20x subconcussive: p = 0.009). There was no interaction of test session and injury.

Figure 5.

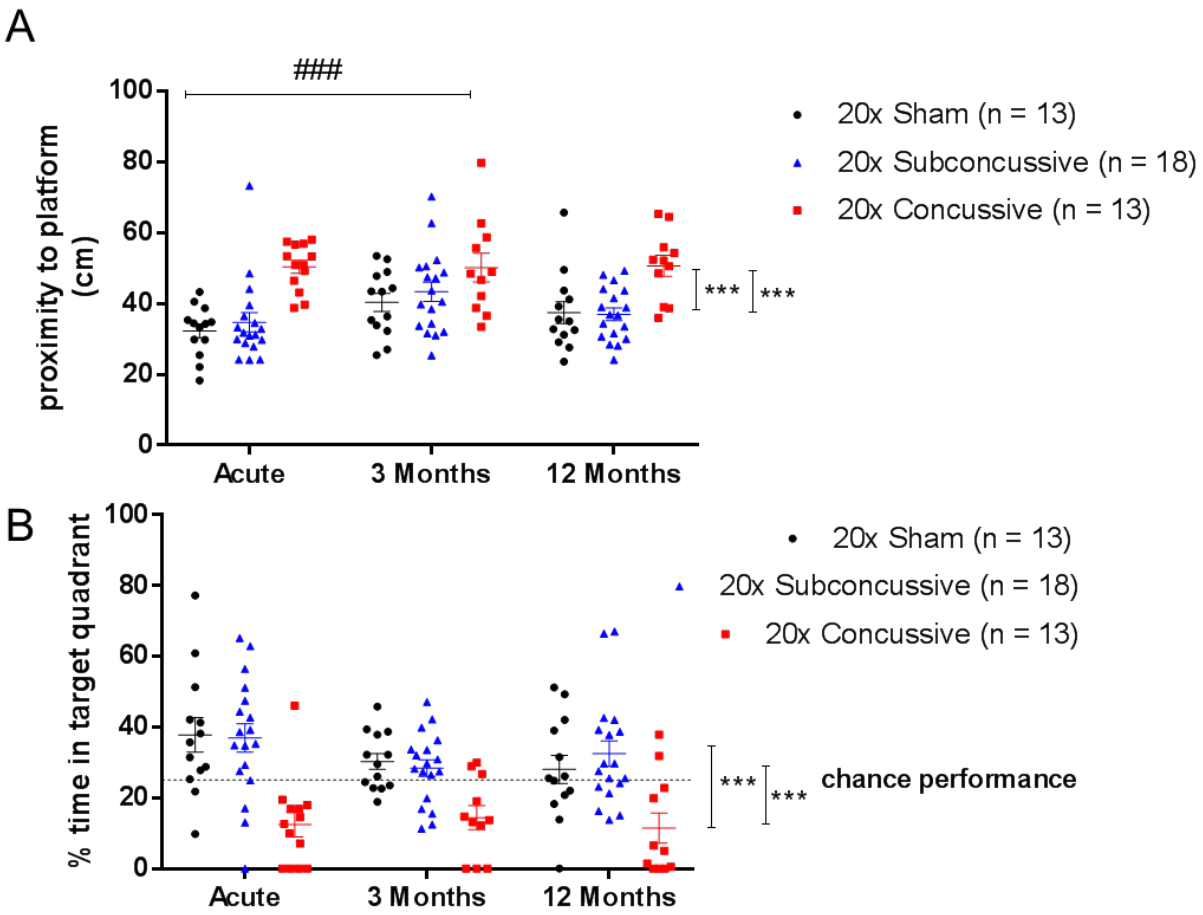


Figure 5. Effects of injury on Morris water maze probe trial performance. A. There was a significant effect of concussive injury on average proximity to platform during probe trial; 20x concussive vs 20x sham: p =0.000124, 20x concussive vs 20x subconcussive: p = 0.000126). There was also an effect of time, where animals were significantly further away from the platform at the 3 month test session (p = 0.000280) relative to the acute session (###). Animals

in the repetitive concussive injury group were on average further away from the location of the target platform. **B.** There was a significant effect of concussive injury on the percent time spent in the quadrant where the platform had previously been placed (20x concussive vs sham: p =0.000124, 20x concussive vs 20x subconcussive: p = 0.000123). Animals in the repetitive concussive injury group tended to spend less than 25% of their time (chance performance, dotted line) in the target quadrant. There was no interaction between test session and injury.

Figure 6.

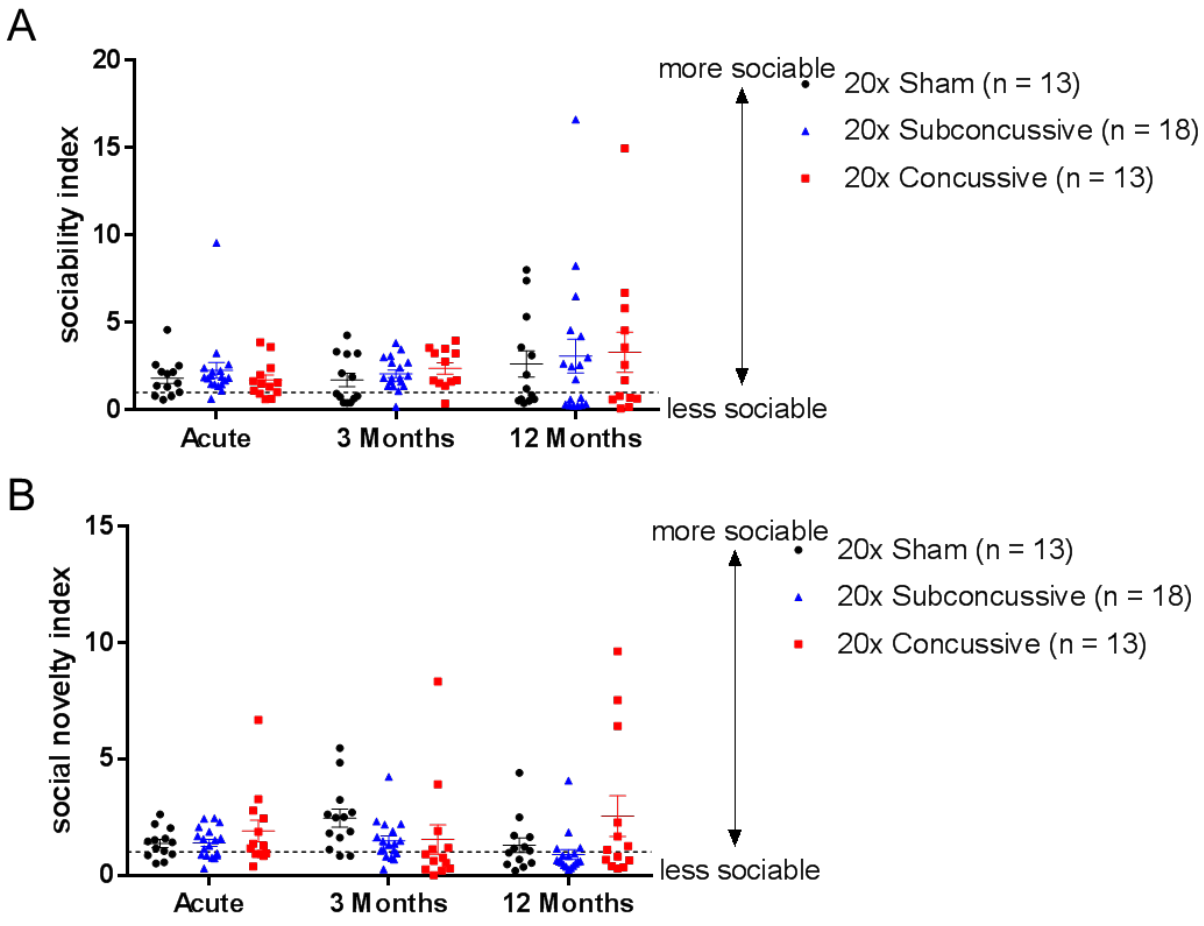


Figure 6. No change in social interaction following injury. A. Injured animals did not show any significant changes in sociability compared to shams. Dotted line indicates a sociability index of one. Points above this line indicate that the animal showed preference for the stimulus mouse rather than the novel inanimate object. **B.** Injured animals did not show any significant changes in social novelty compared to shams. Dotted line indicates a social novelty index of one. Points above this line indicate that the animal showed preference for the novel mouse rather than the stimulus mouse from the previous sociability session.

Figure 7.

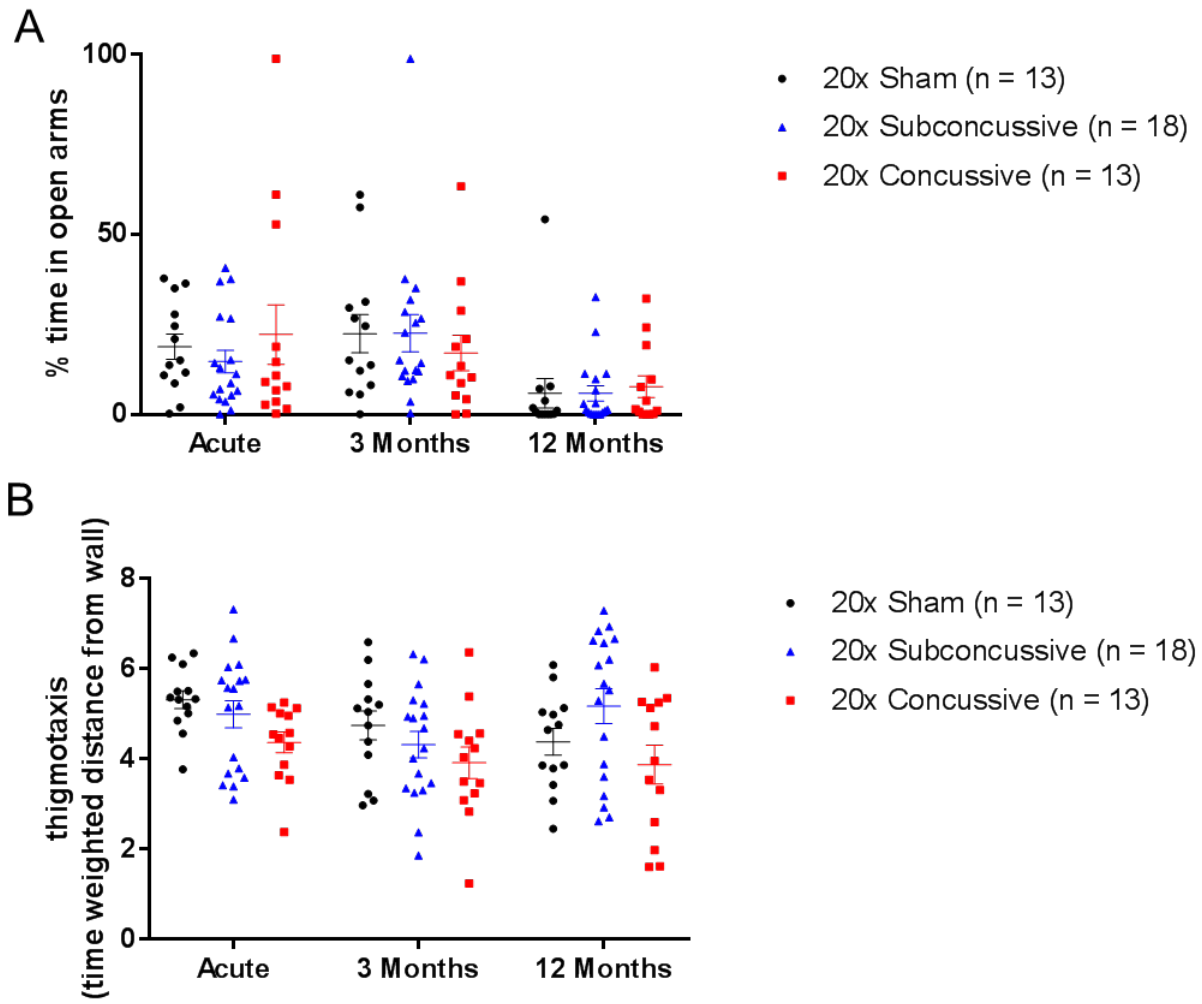


Figure 7. No changes in anxiety related behavior following injury. A. Animals in the 20x concussive and 20x subconcussive did not show any changes in percent time spent in open arms of the elevated plus maze relative to shams. **B.** Injured animals did not show any changes in open field thigmotaxis (time weighted distance from the wall) relative to sham animals.

Figure 8.

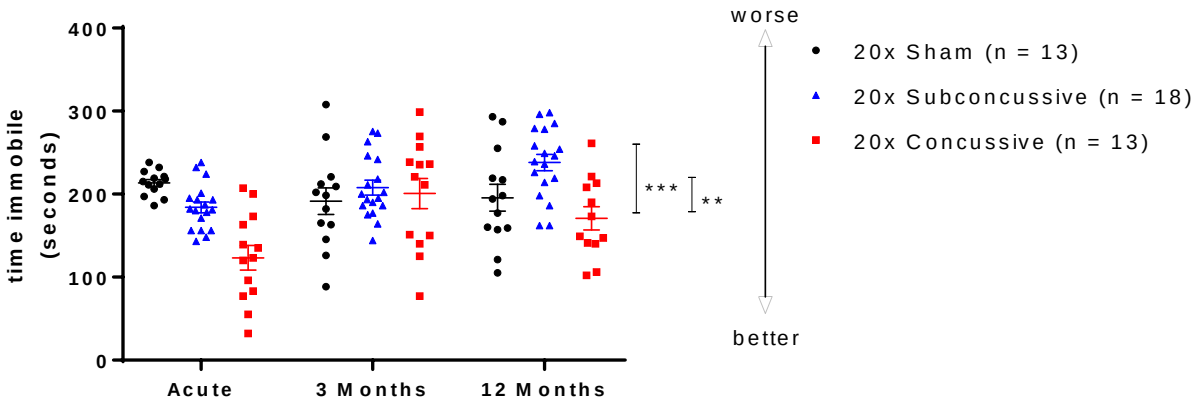


Figure 8. Effects of injury on depressive-type behavior. The tail suspension test was used to assess depressive-type behavior in animals. There was a significant effect of injury (20x concussive vs sham: p = 0.00427, 20x concussive vs 20x subconcussive: p = 0.000212) with less immobility in the 20x concussive group.

Figure 9

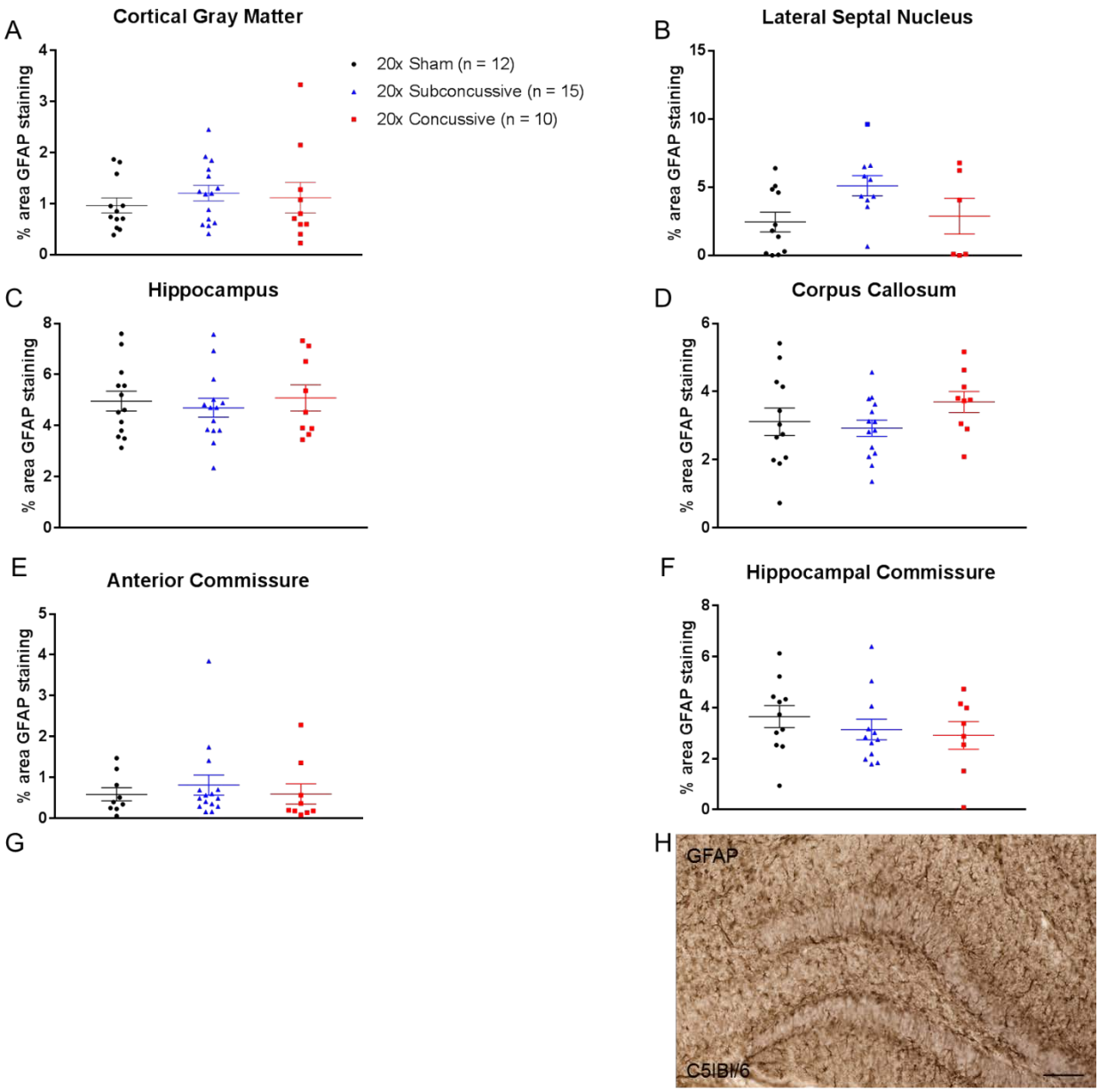
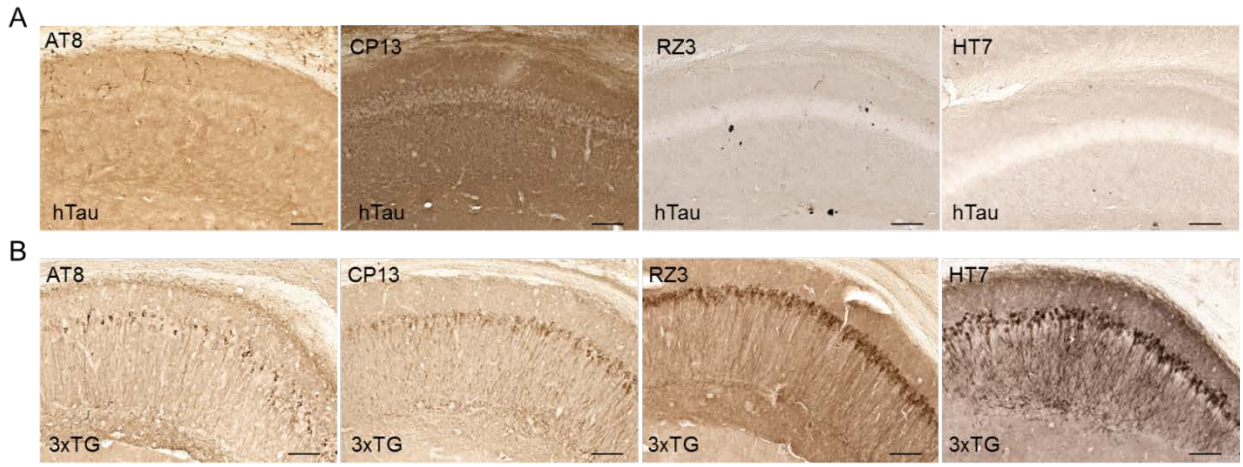


Figure 9. No effects of injury on chronic astrogliosis. A. Percent area of GFAP staining was measured in cortical gray matter. There were no differences in staining between injury groups. **B.** There was no difference in GFAP staining between injury groups in the lateral septal nucleus. **C.** No differences in GFAP staining were detected in the hippocampus **D.** No differences in GFAP staining were detected in the anterior commissure. **E.** There were no differences between injury groups detected in the hippocampal commissure. **F.** No differences in GFAP staining

were detected in the fimbria. **H.** GFAP staining in a positive control wild type mice injured using CCI shows extensive astrogliosis in the hippocampus contralateral to the injury site. Scale bar = 100 microns.

Figure 10.



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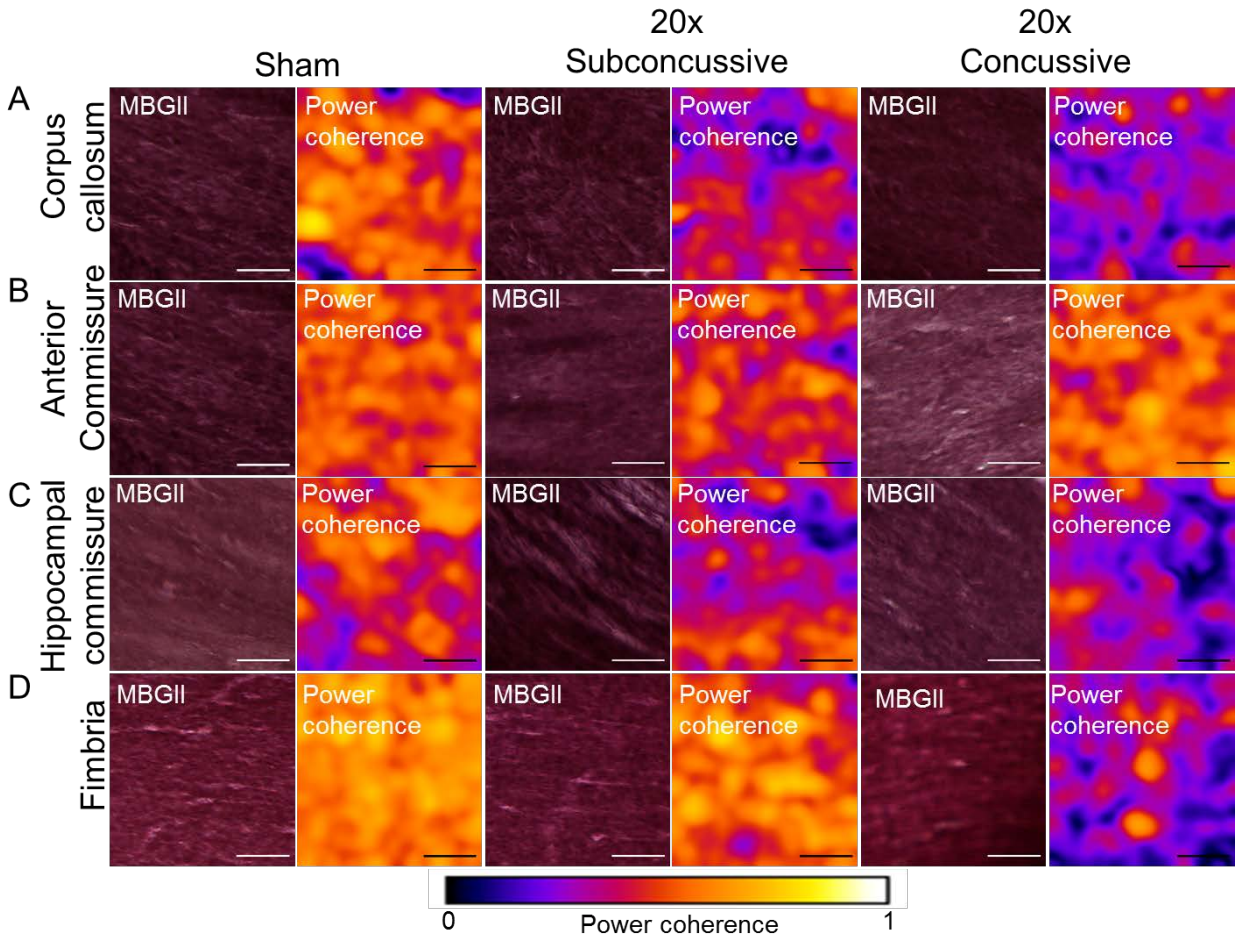

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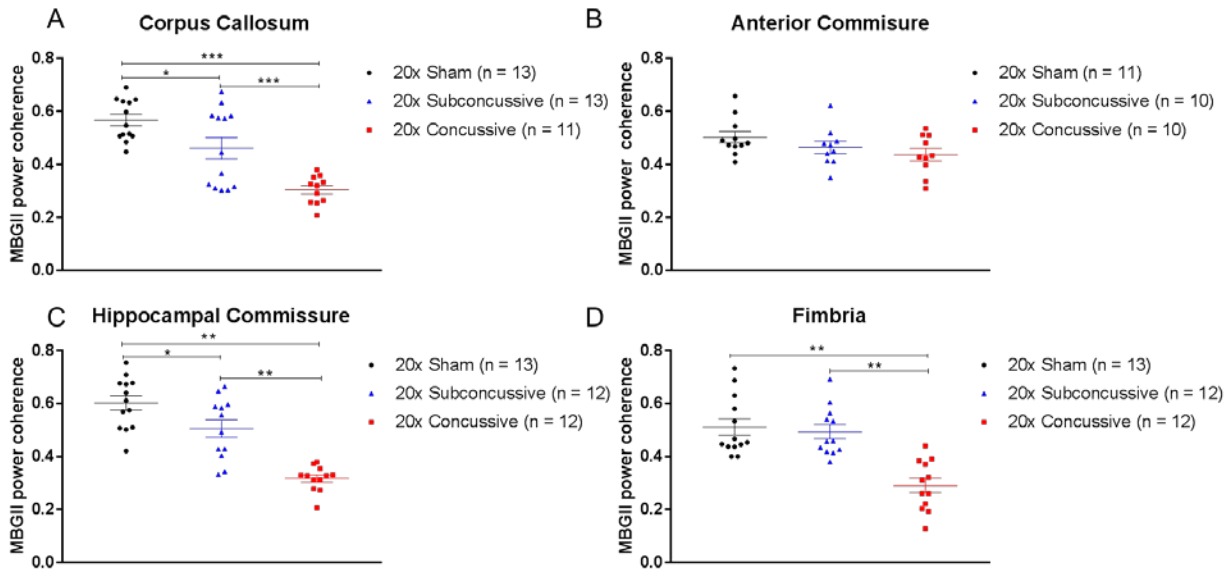

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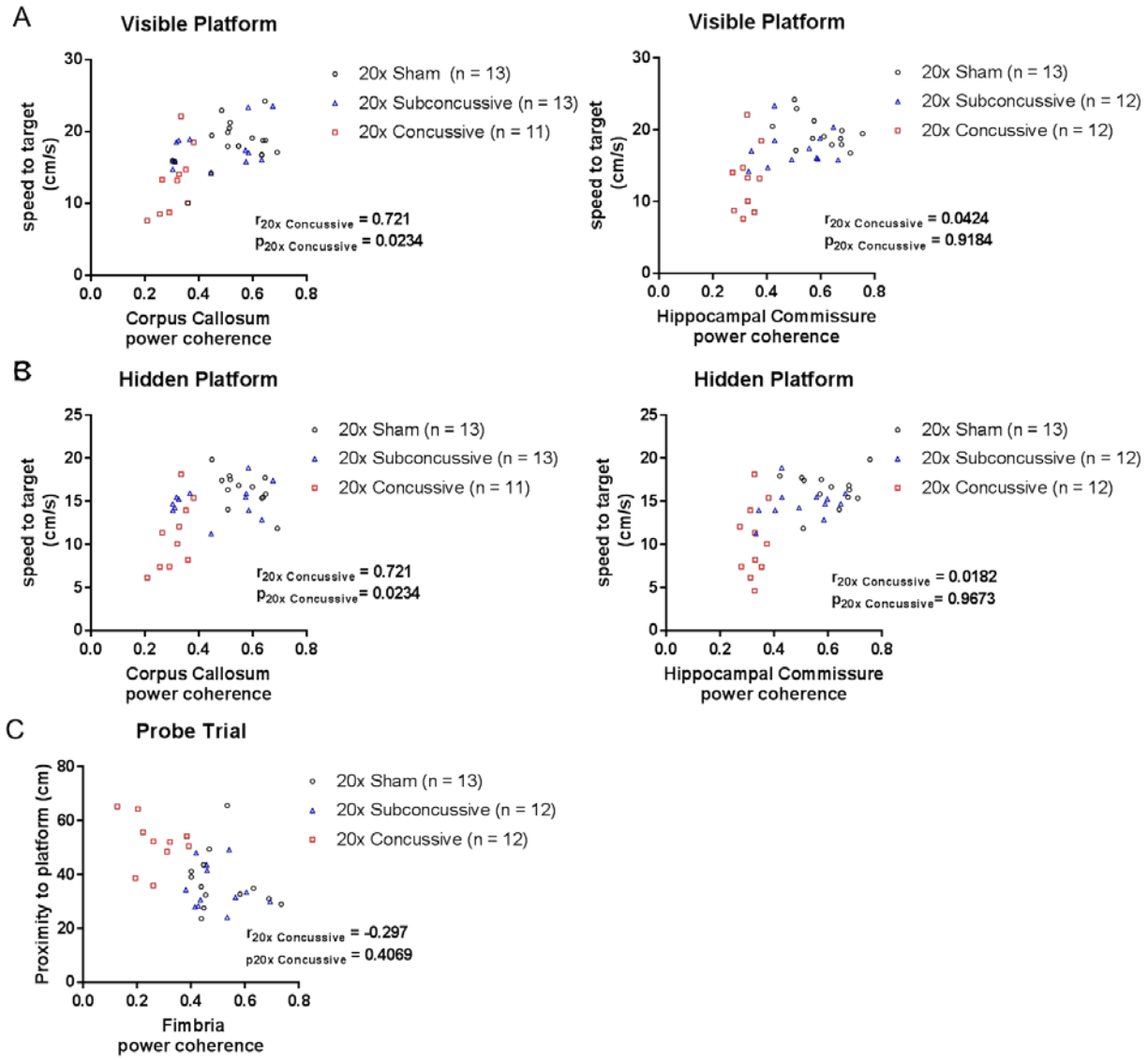

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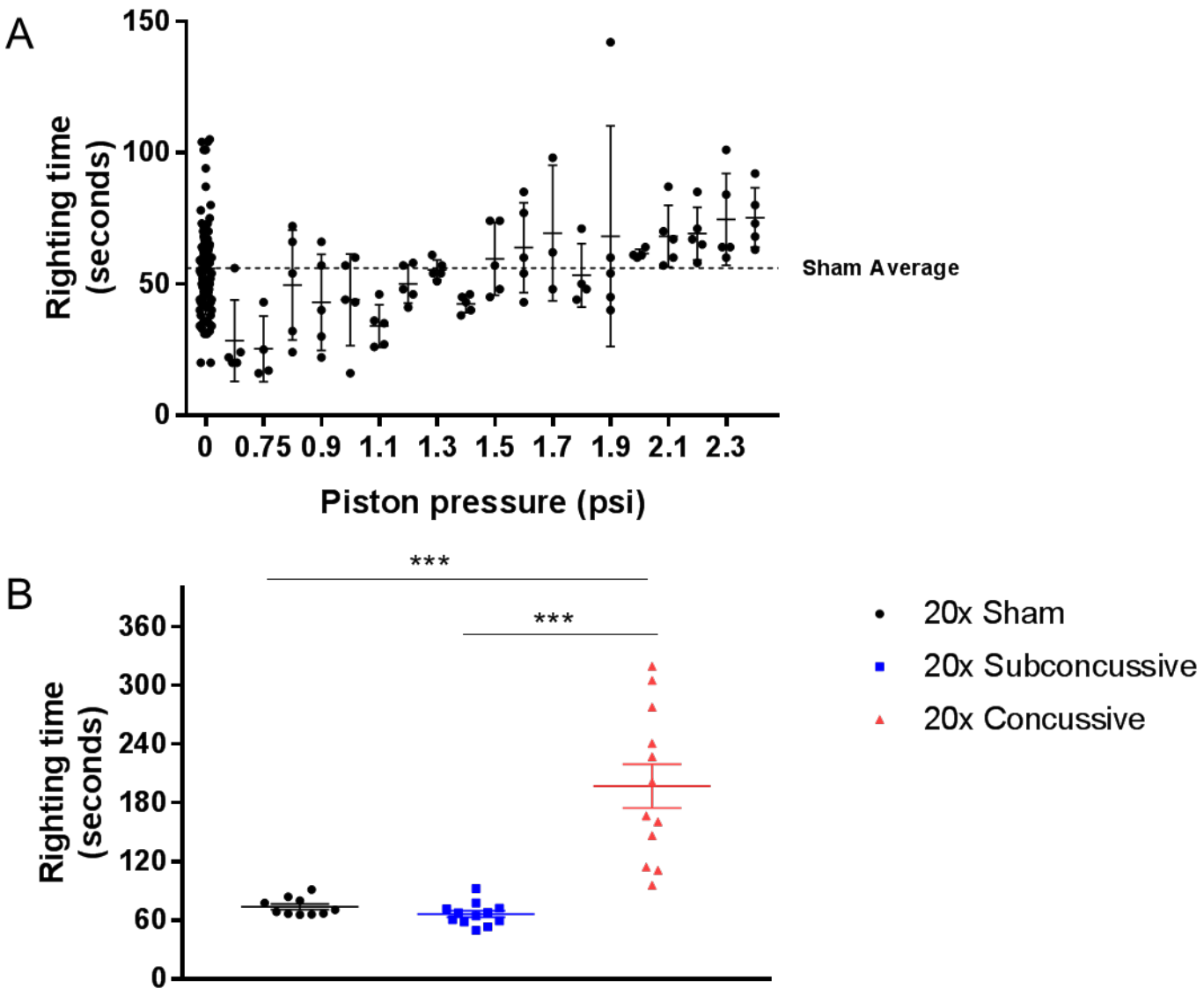
Repetitive concussive and subconcussive injury in a mouse model results in chronic cognitive dysfunction and disruption of white matter tracts

Supplementary Information

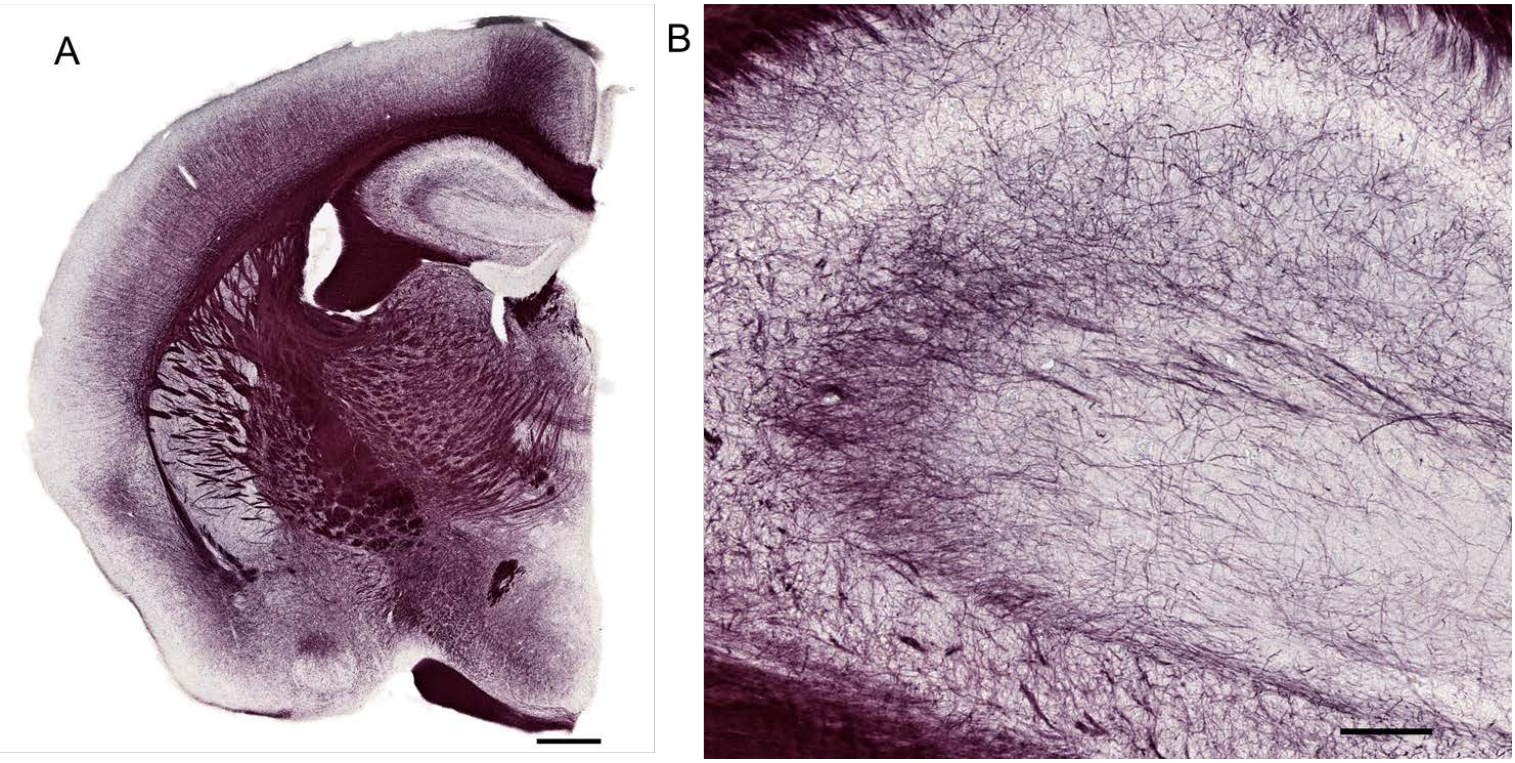
Supplementary Figures

Supplementary Tables

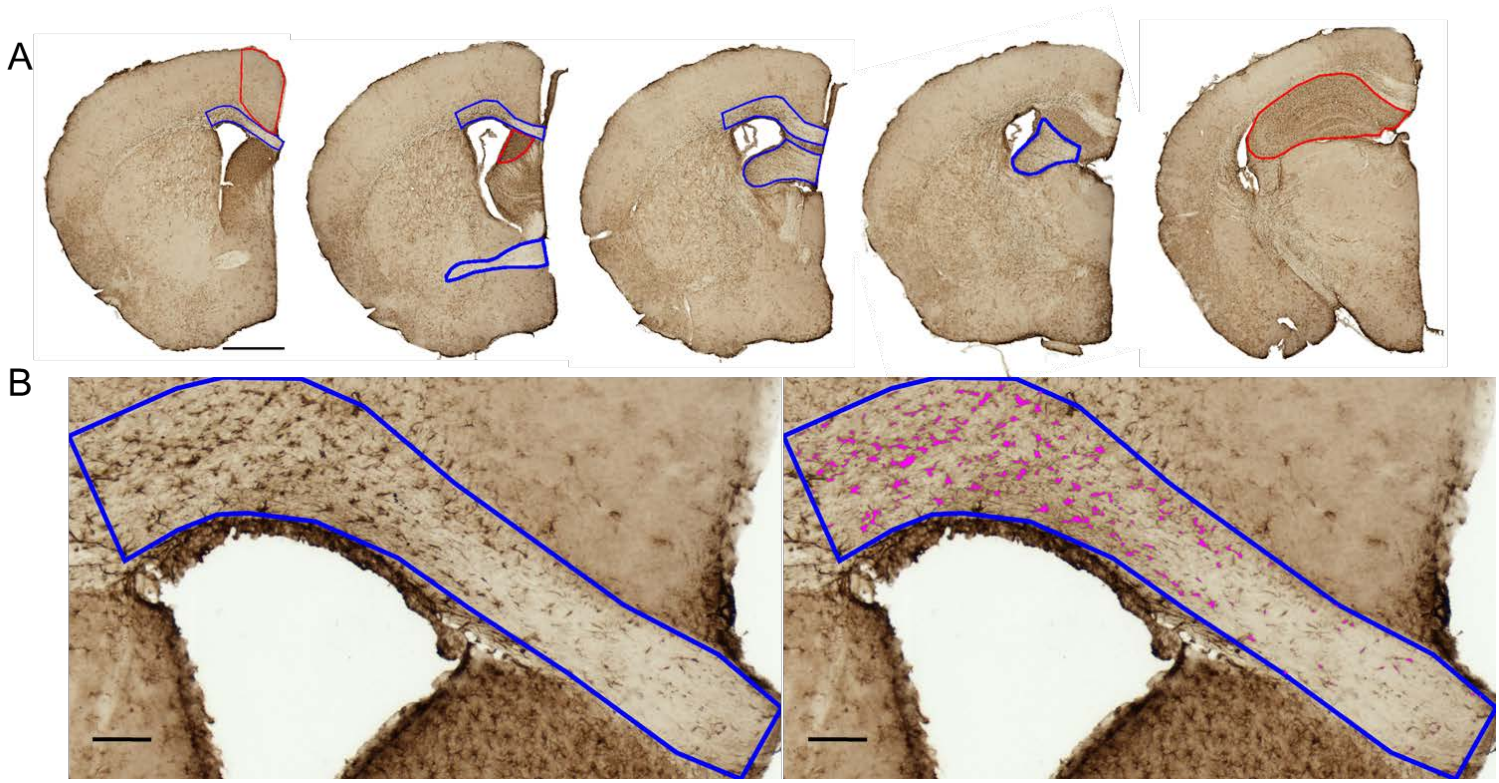
Supplementary References



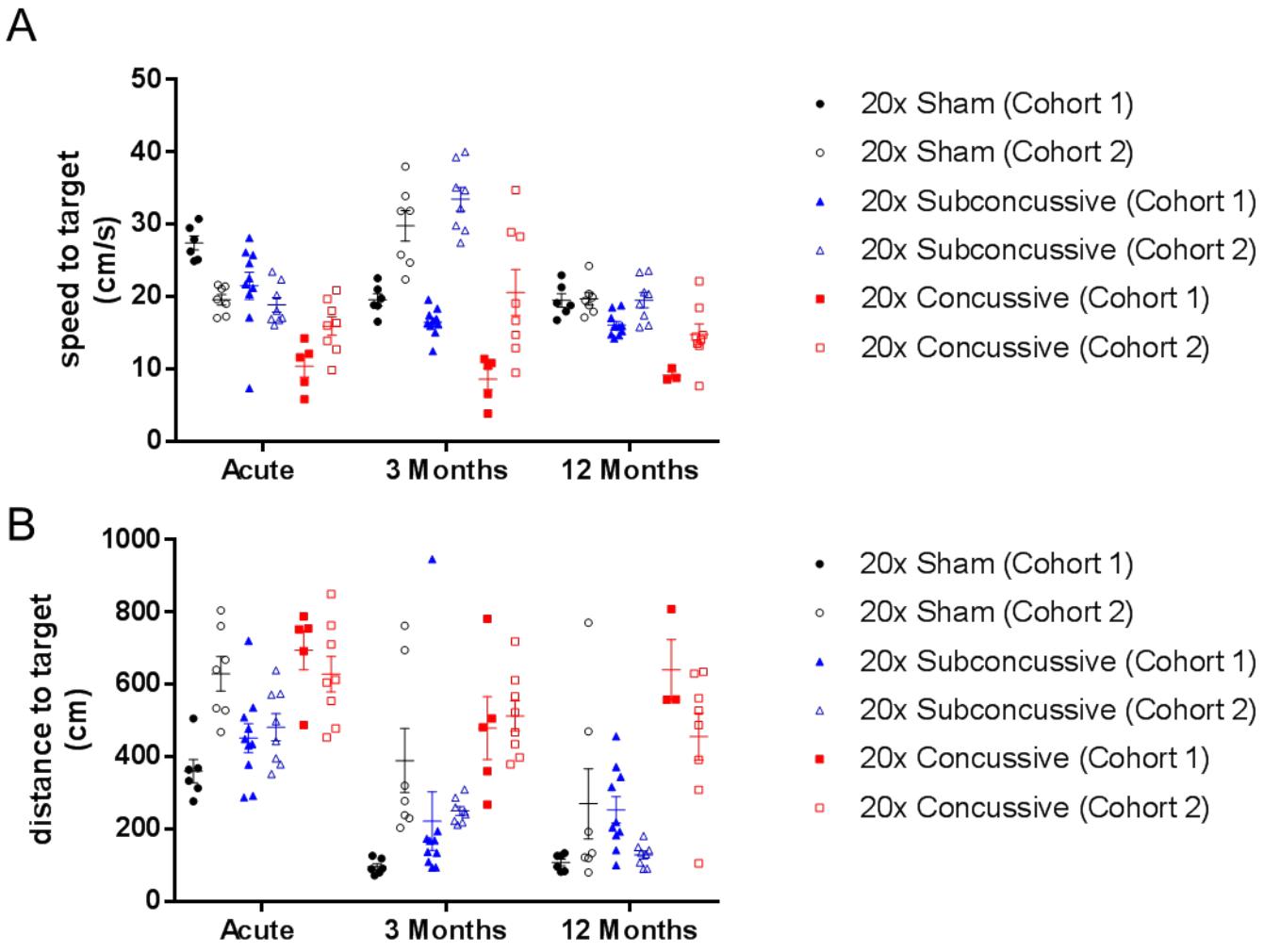
Supplemental Figure 1. Determination of impact thresholds. A. Wild type mice were randomly assigned to receive a single impact with the CHIMERA piston pressure set to a value between 0 (sham) and 2.4 psi. Latency to righting reflex (righting time) was measured following impact for each animal. Mice in groups receiving an impact of 2.0 psi or higher had mean righting times greater than the sham average (56 seconds), with standard deviations that did not overlap with the sham average righting time. **B.** In a separate experiment, mice (n = 10-12 per group) received daily concussive (3.1 psi) or subconcussive (2.0 psi) injuries for twenty consecutive days. Mice in the 20x concussive group had increased righting times averaged over the course of the twenty days relative to shams ($p = 0.000126$) and animals in the 20x subconcussive group ($p = 0.000125$). There was no difference between sham animals and 20x subconcussive animals.



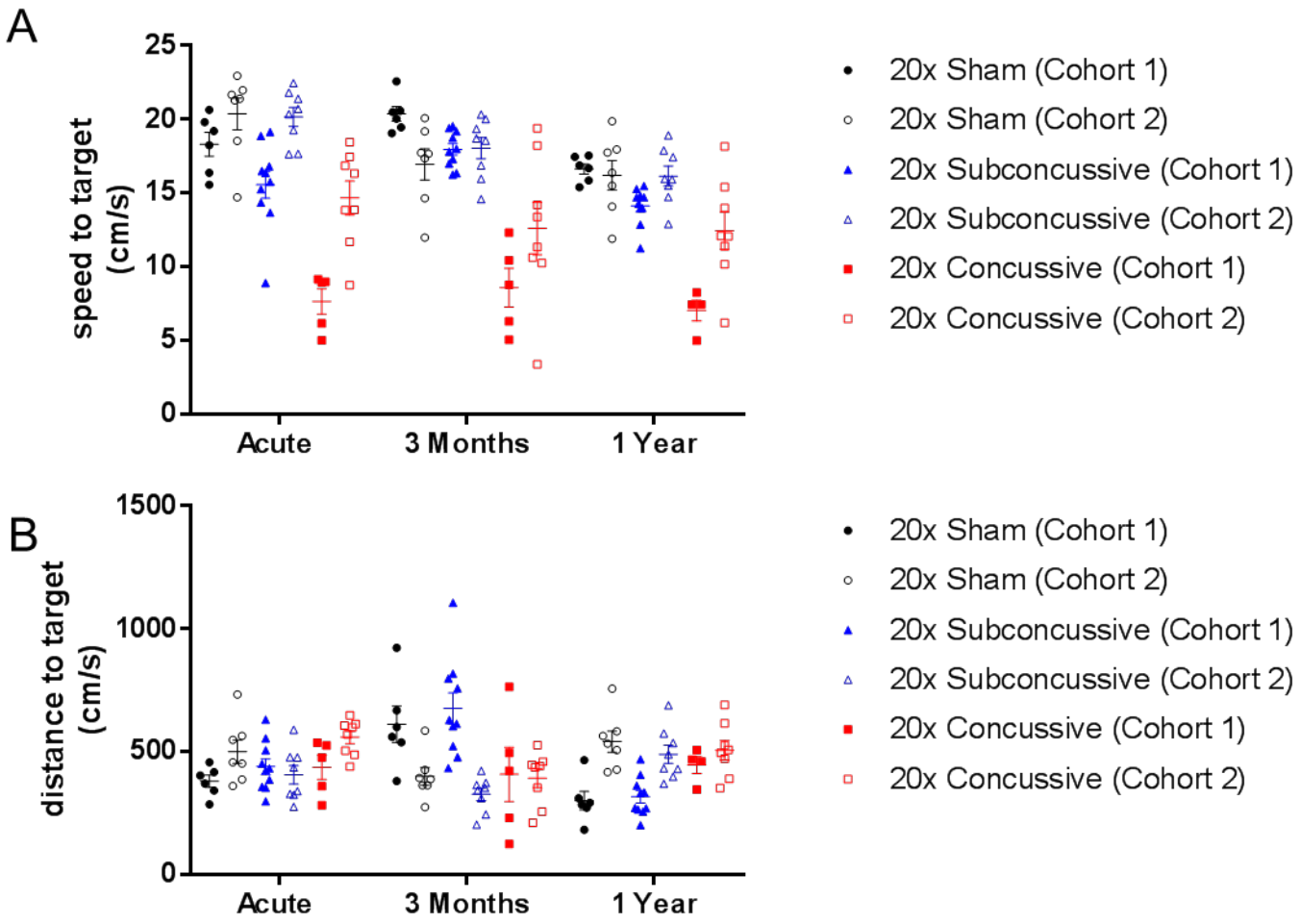
Supplementary Figure 2. Myelin Black Gold II staining. A. 50 µm free floating sections were incubated in Black Gold II solution for 8-12 minutes. Scale bar = 500 microns. **B.** Staining was completed once the mossy fibers of the hippocampus were stained. Scale bar = 100 microns.



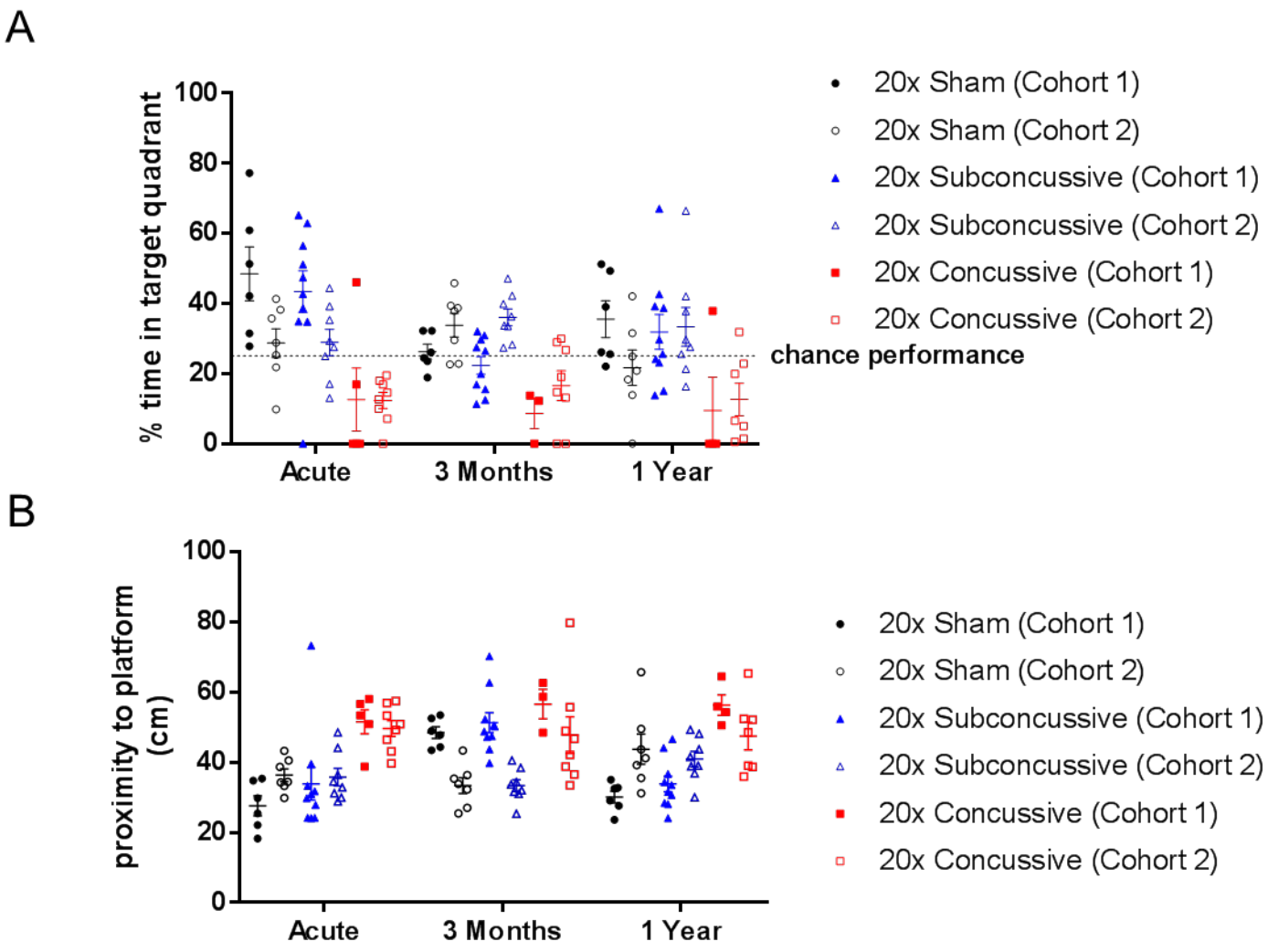
Supplemental Figure 3. Quantification of astrogliosis. A. Regions of interest were drawn to include the corpus callosum, cortical gray matter, lateral septal nucleus, anterior commissure, hippocampal commissure, fimbria, and hippocampus (red line = gray matter, blue line = white matter). Scale bar = 500 microns. **B.** Astrogliosis was quantified by thresholding images of the GFAP stained sections in FIJI, followed by size exclusion to remove edge artifacts and out of focus cell bodies. Scale bar = 100 microns



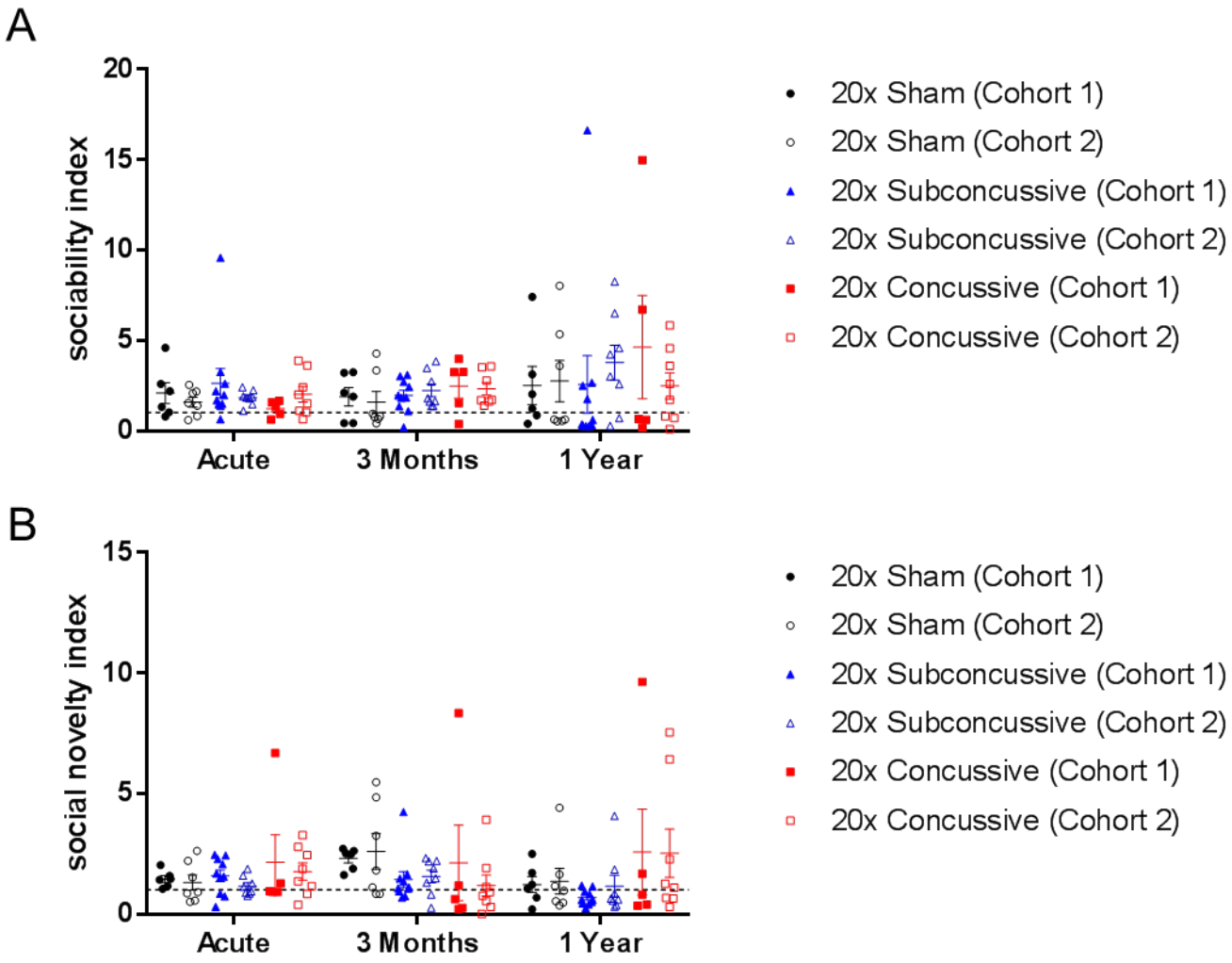
Supplemental Figure 4. Raw data of visible phase of Morris Water Maze. A. Swimming speeds during the visible phase of Morris Water Maze. Speeds represent the mean for each subject across the three days of visible platform for each test session. **B.** Distance to the target platform during the visible phase of Morris Water Maze. Distances represent the mean for each subject across the three days of visible platform for each test session.



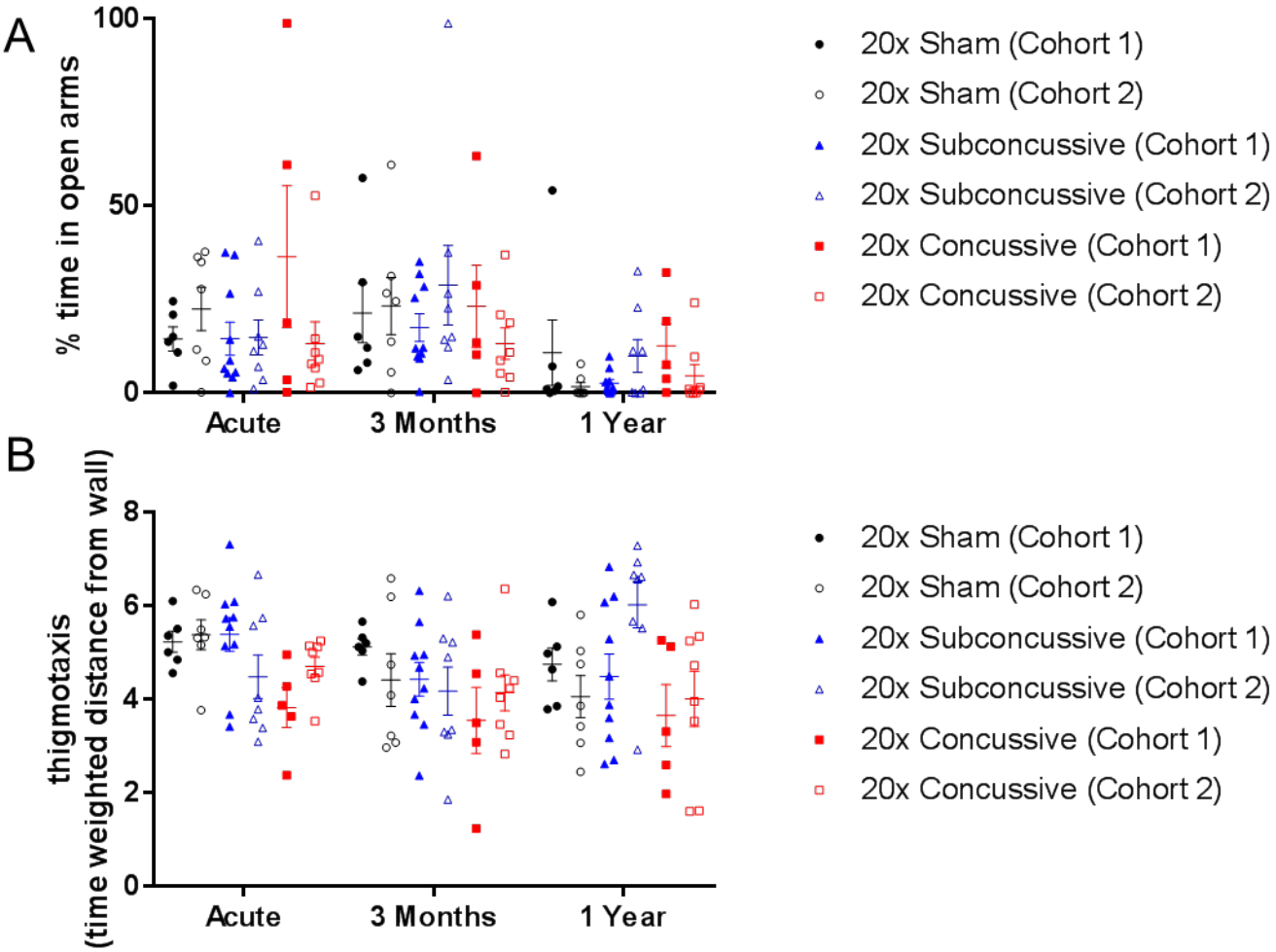
Supplemental Figure 5. Raw data of hidden phase of Morris Water Maze. A. Swimming speeds during the hidden phase of Morris Water. Speeds represent the mean for each subject across the four days of hidden platform for each test session **B.** Distance swum to platform during the hidden phase of Morris Water Maze. Distances represent the mean for each subject across the four days of hidden platform for each test session.



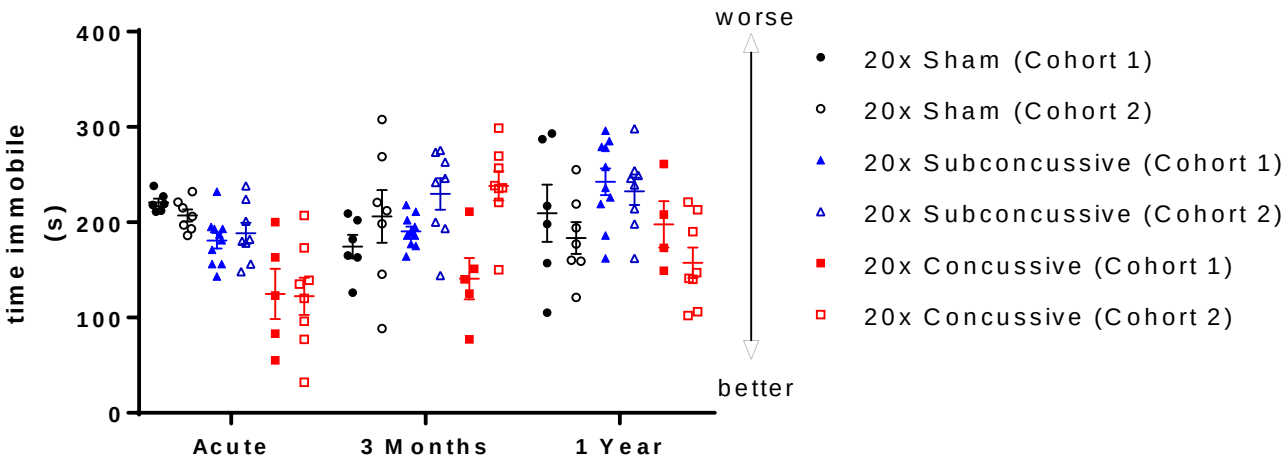
Supplemental Figure 6. Raw data of probe trial performance. A. Raw data of percent time spent in the target quadrant during probe trial. Dotted line indicates chance performance, where mice spending less than 25% of their time in the target quadrant show impaired memory. **B.** Raw data of the mean proximity to the location of where the platform had been placed during the preceding hidden platform phase. Increased proximity to platform indicates worse performance and impaired memory of the platform location.



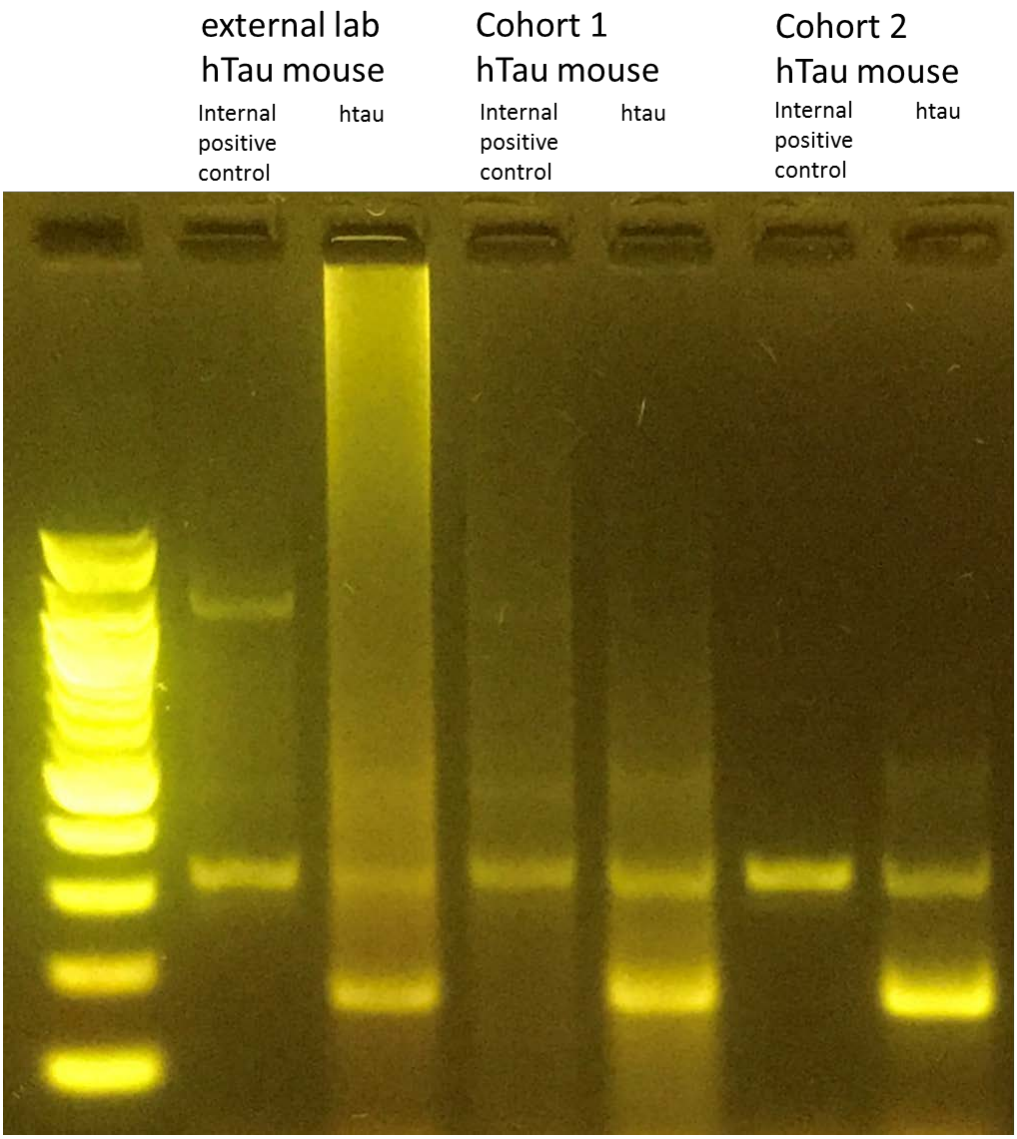
Supplemental Figure 7. Raw data of social interaction task. A. Raw data of sociability index during the three chamber social interaction test. Sociability index was calculated as the ratio of time interacting with the stimulus mouse to time spent with the novel object. The dotted line marks an index of one. Points above this line indicate that the animal preferred to interact with the stimulus mouse. **B.** Raw data of social novelty index during the three chamber social interaction test. Social novelty index was calculated as the ratio of time interacting with the novel mouse to time spent with the first stimulus mouse. The dotted line marks an index of one. Points above this line indicate that the animal preferred to interact with the novel mouse.



Supplemental Figure 8. Raw data of elevated plus maze and open field maze. A. Percent time spent in the open arms of the elevated plus maze. **B.** Raw data of open field maze thigmotaxis, measured as the time weighted distance from the maze wall.



Supplemental Figure 9. Raw data of tail suspension test. Raw data of time immobile during a six minute tail suspension test was measured for each animal during each test session.



Supplemental Figure 10. PCR genotyping of hTau mice. PCR was performed by DNA extraction from fresh-frozen brain tissue of one hTau mouse randomly selected from each cohort of animals purchased from Jackson Laboratory. As a positive control, tail DNA from an hTau mouse was obtained from an independent investigator. The animals from each cohort tested positive for the hTau transgene.

Authors	Animal type	Age at time of injury	Injury model	Number of injuries	Time between injuries	Acute behavioral outcomes	Acute pathology	Chronic behavioral outcomes	Chronic pathology
Kanayama et al 2001[1]	Rat	N/A	FPI (1.0 atm)	7	24 hours	N/A	Accumulation of MAP2 and p-NFH spreading to the contralateral cortex and hippocampus. pTau immunoreactivity in cortical neurons	N/A	N/A
Creeley et al 2004[2]	C57BL/6	7-8 weeks	Weight drop	3	24 hours	Impaired spatial learning during MWM	Silver staining abnormalities in olfactory and optic tracts	N/A	N/A
Shitaka et al 2011[3]	C57BL/6	2-3 months	Closed head CCI	2	24 hours	MWM deficits	Abnormal APP swellings, argyrophylic abnormalities	7 weeks; MWM deficits	Persistent microglial activation, white matter silver staining
Ojo et al 2013[4]	hTau mice	18 months	Closed head CCI	5	48 hours	N/A	Increased ptau immunoreactivity no perivascular or astroglial tau. Increased microglial and astrocytic reactivity	N/A	N/A
Mannix et el 2013[5]	C57BL/6	2-3 months	Weight drop	7	24 hours	Impaired balance and MWM deficits	N/A	6 months: Impaired balance and MWM deficits	Elevated microgliosis and astrogliosis

Petraglia et al 2014a/2014b[6, 7]	C57BL/6	3 months	Closed head CCI with acceleration/deceleration (no anesthesia)	42	2 hours	Elevated NSS, increased anxiety like behavior, MWM deficits, sleep disturbances, depressive behavior	Reactive astrocytosis elevated ptau immunoreactivity	Increased risk taking behavior at 6 months, persistent MWM deficits	Astrogliosis in cortex 6 months post injury, persistent microgliosis, elevated ptau immunoreactivity
Luo et al 2014[8]	C57BL/6	2-3 months	Closed head CCI	3	24 hours	Spatial learning and memory deficits	N/A	6 months post injury; spatial learning and memory deficits	6 months post injury; increased astrogliosis, increased pTau immunoreactivity
Turner et al 2015[9]	Sprague-Dawley rats	N/A	Blast injury	6	48 hours	Increased open arm time in EPM, MWM deficits	Increased ptau immunoreactivity	N/A	N/A
Bajwa et al 2016[10]	C57BL/6	3 months	Unrestrained closed head CCI	2	72 hours	No differences	N/A	90 days post injury; increased depressive like behavior Reduced social activity	N/A
Ojo et al 2016[11]	hTau mice	3 months	Closed head CCI	24 or 32	72 or 96 hours	N/A	N/A	6 months post injury; impaired MWM performance, increased risk taking behavior	Increased total tau levels, mild increase in ptau.
Chen et al 2017[12]	C57BL/6	12 weeks	0.5J CHIMERA	3	24 hours	Motor deficits, MWM deficits	APP deposition, reduced by 1 month	6 months post injury; impaired MWM deficits	Increased GFAP, Iba1 levels

Supplemental Table 1. Preclinical animal models of repetitive traumatic brain injury. Multiple groups have implemented repetitive head injury paradigms in rodent models (mouse and rat) in an attempt to study chronic phosphorylated tau pathology commonly found in human patients diagnosed with chronic traumatic encephalopathy.

Dataset	Normally distributed? (Shapiro Wilk ($p < 0.05$)	Transformation applied	Effect of Cohort
Three chamber social interaction: sociability index	No	Rank (no ties)	No
Three chamber social interaction: social novelty index	No	Rank (no ties)	No
Open field maze: thigmotaxis	Yes	N/A	No
Elevated plus maze: % time in open arms	No	Rank (no ties)	No
Morris Water Maze visible platform: speed to target	No	Averaged across test session	No
Morris Water Maze visible platform: distance to target	No	Averaged across test session	No
Morris Water Maze hidden platform: speed to target	No	Square root	No
Morris Water Maze hidden platform: speed to target	No	Rank transformed averaged across test session (no ties)	No
Morris Water Maze probe trial: proximity to platform	No	Inverse (1/x)	No
Morris Water Maze probe trial: % time in target quadrant	No	Rank	No
Astrogliosis: % area GFAP staining	Yes	N/A	No
White matter integrity: power coherence	Yes	N/A	No

Supplemental Table 2. List of transformations applied for statistical analysis of data. Datasets where the residuals were not normally distributed (Shapiro Wilkes, $p<0.05$), were transformed so that residuals would be normally distributed. A two way (repeated measures for behavioral data) ANOVA was then used to test for effects of injury and cohort. Once transformed, there was no effect of cohort, allowing us to collapse the datasets for the two cohorts. Post-hoc Tukey testing was then used to test for significant effects of repetitive subconcussive and concussive injury.

Behavioral/Histological Test	Effect Size (η^2_p)
Latency righting reflex	0.349
MWM visible platform: swim speed	0.624
MWM visible platform: swimming distance	0.588
MWM hidden platform: swim speed	0.663
MWM hidden platform: swimming distance	0.564
MWM probe trial: proximity to platform	0.570 (Injury) 0.167 (Time)
MWM probe trial: % time in target quadrant	0.566
Three chamber social interaction: sociability	0.0159
Three chamber social interaction: social novelty	.0860
Open field maze: thigmotaxis	0.0243
Elevated plus maze: % time in open arms	0.00102
Tail suspension: time immobile	0.412
Astrogliosis: % area GFAP staining cortical gray matter	0.125
Astrogliosis: % area GFAP staining lateral septal nucleus	0.368
Astrogliosis: % area GFAP staining hippocampus	0.00868
Astrogliosis: % area GFAP staining corpus callosum	0.0156
Astrogliosis: % area GFAP staining anterior commissure	0.0138
Astrogliosis: % area GFAP staining hippocampal commissure	0.0126
Astrogliosis: % area GFAP staining fimbria	0.0778
White matter integrity: power coherence corpus callosum	0.644
White matter integrity: power coherence anterior commissure	0.144
White matter integrity: power coherence hippocampal commissure	0.687
White matter integrity: power coherence fimbria	0.527

Supplemental Table 3. List of effect sizes for ANOVAs. The effect sizes (η^2_p) were calculated for each behavioral and histological parameter assessed.

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